PHOTOSYNTHESIS

ROBERT HILL and
C. P. WHITTINGHAM

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PHOTOSYNTHESIS

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Photosynthesis

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PREFACE

In this book we have attempted to give a brief account of the biochemistry and the physiology of photosynthesis in such a way as to provide an introduction to present researches in this field.

We are grateful to many of our colleagues for their help; in particular we are indebted to Professor G. E. Briggs, F.R.S., whose teaching has greatly contributed to Chapters 2 and 4, and who has allowed us to select freely from his unpublished lecture notes, and to Professor E. J. Maskell, F.R.S., who kindly criticized the manuscript. We wish to thank Miss C. Lambert who prepared the drawings.

R. H.
C. P. W.

CAMBRIDGE
November 9th, 1953

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CHAPTER I

INTRODUCTION

From the time of Aristotle until the eighteenth century it was generally supposed that plants derived their nourishment from the soil. This view was questioned by Stephen Hales in 1727, who suggested that plants took some part of their nourishment from the air and he added the remark that light also probably played some part in the process. Further progress was possible only after chemists had learnt to distinguish the different gases which constitute air and it was Priestley, the discoverer of oxygen, who made one of the first important contributions to the study of photosynthesis. In 1771 he found that green plants had the ability to 'restore' air which had been made bad, as the result of combustion or by the respiration of animals, to a condition in which it could again support combustion and life. Ingen Housz in 1772 showed that light, as well as the green plant, was concerned in the purification of air and that in the dark, plants, just like animals, caused air to become 'foul'. This discovery led Ingen Housz to exaggerate the danger of poisoning which might arise when plants are kept in the dark in houses and to the tradition that plants or portions of plants with flowers should be removed from the bedroom at night. Senebier in 1782 demonstrated an accelerating effect of the 'noxious' gas produced by animals and by plants in the dark ('fixed air', or as we now call it carbon dioxide) upon the production by plants of 'dephlogisticated air' (i.e. oxygen) in the light. The introduction of quantitative experiments allowed de Saussure to show in 1804 that the gain in weight of a plant during a period of photosynthesis plus the weight of the oxygen evolved exceeded the weight of the carbon dioxide consumed. He suggested that the difference must be due to an uptake of water. Thus by the early nineteenth century the green plant had been shown to be an autotrophic (from Gk. *auto-*, self, and *trophe*, nourishment) organism. It can be grown without soil in water containing the essential mineral salts provided carbon dioxide and adequate illumination are supplied.

These observations can be easily demonstrated today using the simplest apparatus. The gas produced upon the illumination of a fresh water alga placed in bicarbonate solution can be shown to rekindle a glowing splint. Many of us must have unconsciously repeated Priestley's experiment when we were children and placed green water weeds in our jars containing tadpoles! The production of organic matter by the illuminated green leaf is most easily demonstrated when starch is formed. After removal of the green pigment by treatment with alcohol and subsequent washing, starch can be detected by staining the leaf with iodine. In this way it can be shown that starch accumulates in many leaves during the day time, i.e. when both light and carbon dioxide are available. If the leaf is suitably masked during illumination it will be seen that the starch is confined to the areas exposed to the light. During the night the starch tends to disappear from the leaf being in part used in leaf respiration and in part translocated in the form of sugars, to other parts of the plant.

The products of photosynthesis, oxygen and organic compounds, are consumed in respiration, so that the two processes may be summarized in one equation, the direction of the change indicating which process is predominating.

$$\text{Organic compounds} + O_2 \underset{\text{Photosynthesis}}{\overset{\text{Respiration}}{\rightleftharpoons}} CO_2 + H_2O \qquad (1.1)$$

When we attempt to measure photosynthesis in the living plant we observe only the net process—the 'apparent' photosynthesis. We may, however, measure the rate of respiration in the dark, preferably both before and after the light period, and add the mean of these values to the 'apparent' rate of photosynthesis and thus obtain an estimate of the 'real' photosynthesis. This procedure, which assumes that respiration continues in the light at the same rate as in the dark, may not always be justified. The experimental evidence so far available is not adequate to decide the question. With brightly illuminated leaves, however, the rate of 'apparent' photosynthesis may be as much as fifty times the rate of respiration in the dark so that the correction to 'real' photosynthesis is here relatively very small. Under field conditions the rate of photosynthesis in normal daylight is 5 to 10 times the respiration rate. Under weak illumination respiration may predominate even in the light and the estimate of 'real' photosynthesis will largely depend on the value adopted for the respiration rate. At some intermediate light intensity, the compensating light intensity, photosynthesis and respiration will balance exactly and there will be no net gas exchange.

The reaction represented by equation (1.1) is in practice not directly reversible by increasing the concentrations of carbon dioxide and water relative to those of oxygen and sugar; under any realizable concentrations of reactants or products very considerable energy is required to make the over-all reaction proceed in the direction of synthesis. During photosynthesis light energy is converted into chemical energy in the form of the ultimate photosynthetic end products. These in turn form the primary reactants in respiration, so that from one point of view the biochemistry of plant respiration and of the metabolic processes with which it is linked is a study of the chemical reactions involved in the distribution of energy originally derived from light.

In equation (1.1) the product of photosynthesis has been written as organic compounds. If this is simply regarded as carbohydrate the amount of carbon dioxide consumed will equal that of oxygen produced.

$$CO_2 + H_2O \rightleftharpoons \frac{1}{n}(CH_2O)_n + O_2 \qquad (1.2)$$

The ratio of the two quantities (CO_2/O_2) called the assimilatory quotient, is in fact found to be near to unity in a very wide range of plants and under various conditions (Table 1.1). In a leaf detached from the plant the product of photosynthesis is frequently stored as starch; in a complete growing plant or culture of micro-organisms, e.g. *Chlorella*, the ultimate result of photosynthesis is an increase in cell material. This material includes protein and lipids as well as carbohydrate. The assimilatory quotient for the formation of protein has an average value of — 0·8 and that for lipid — 0·7. Small departures from unity for the assimilatory quotient for the over-all process may thus be attributed to the formation of a greater or lesser amount of products other than carbohydrate.

TABLE 1.1

THE ASSIMILATORY QUOTIENT IN A VARIETY OF PLANTS

Measurements made with a high light intensity and a high concentration of carbon dioxide. (*From Willstätter and Stoll* (1918), *Barker* (1935), *and the authors' data*)

Plant		Assimilatory quotient
Sambucus nigra	(leaf)	−0·96 to −1·00
Pelargonium zonale	,,	−0·98 to −0·99
Ilex aquifolium	,,	−1·00
Leucobryum glaucum	,,	−0·99
Chlorella pyrenoidosa	(culture)	−0·90 to −0·98
Nitzschia closterium	,,	−0·96

It can be shown experimentally that a plant can grow to maturity when supplied with a single carbohydrate as the sole organic carbon source and this would suggest that a substance equivalent to a simple sugar, triose or hexose, might be the primary product of photosynthesis. Albino seedlings, which are devoid of chlorophyll, will grow only so long as their initial reserve material lasts, and when this is exhausted they die. If, however, these seedlings are grown under sterile conditions in a medium containing a single carbohydrate the plants continue to grow and reach maturity.

Photosynthesis results in the production of the order of 10^{11} tons of organic carbon per year (Rabinowitch, vol. 1). From this figure it can be calculated that on the average each carbon dioxide molecule must be incorporated into plant structure every 200 years and that the oxygen of the air is renewed approximately every 2,000 years.

Equation (1.1) expresses the qualitative facts of plant respiration and photosynthesis. Further analysis of the two processes became possible only when the quantitative relationships of plant metabolism were studied. For such investigations it was found necessary to investigate cells from many types of plant, from the more specialized of the higher plants to the simpler unicellular forms represented by the algae and bacteria. Each type offered certain advantages in the investigation of a particular aspect of photosynthesis. But before such data could be analysed quantitatively the effect of the vast differences in physical structure had to be taken into consideration. This is the subject of the second chapter of this book which is concerned with plant structure as it affects the study of photosynthesis. In the fourth chapter the general relations between the rate of photosynthesis and various environmental factors are considered and we shall see how important plant structure may be in determining a relationship such as that between rate of photosynthesis and the external concentration of carbon dioxide. Physiological studies led to a formulation of the mechanism of photosynthesis in general terms which was subsequently interpreted in chemical terms as the result largely of work with photosynthetic bacteria. This is discussed in Chapter 5. The subsequent chapters discuss more recent researches in biochemistry which are relevant to the problem of photosynthesis and which may ultimately permit the formulation of the mechanism of photosynthesis in specific chemical terms.

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CHAPTER 2

PLANT STRUCTURE IN RELATION TO PHOTOSYNTHESIS

THE structure of the plant will determine the accessibility of the reactant carbon dioxide to the assimilatory cells. For synthesis of one gram in the form of hexose the land plant consumes carbon dioxide equal to that in 2,500 litres of air; measurements indicate that, at the best, the plant utilizes only two thirds of the carbon dioxide of the air and thus the actual volume of air required is greater than this. The concentration of carbon dioxide in the atmosphere is small compared with that of oxygen (0·03% against 21·0%); yet the rate of photosynthesis of a land leaf under natural conditions may be some 10 times greater than the rate of respiration. The rate of these processes may under any set of conditions be determined by either physical or chemical factors. In this chapter we shall consider only the physical factors affecting the supply of reactant to the green cells, e.g. the diffusion of carbon dioxide into the plant.

The diffusion path for carbon dioxide into the cells of the leaf of a higher plant is complex. Carbon dioxide diffuses from the air outside the leaf up to and through the stoma, across the intercellular space, and then through the wet cell wall up to the centre where reaction occurs. The rate of diffusion is proportional to the difference in concentration of carbon dioxide between the two ends of the path, to the area of the path, and inversely proportional to the length of path. It is also directly proportional to the diffusion constant, a characteristic of the substance diffusing and of the medium through which diffusion occurs.

$$\frac{\delta S}{\delta t} = -KA\frac{\delta C}{\delta x} \qquad (2.1)$$

where K is the coefficient of diffusion, A is the area of path
δC is the difference in concentration in a distance δx
δt is a small time interval during which δs is the net quantity of substance transferred.

VARIATION IN THE DIFFUSION PATH FOR DIFFERENT PLANT STRUCTURES

Organisms such as the unicellular algae and bacteria possess the simplest diffusion path, carbon dioxide entering through the whole surface of the plant. In the case of water plants the medium is buffered due to the presence of bicarbonate ions, but the concentration of free carbon dioxide will be less than that in air and the volume necessary for the supply of 1 gm. of assimilate will be slightly greater but of a similar order to that for air. For an alga of small dimensions, e.g. *Chlorella*, the diffusion path is short and, assuming the diffusion constant of carbon dioxide through the cell wall to be of the same order of magnitude as for diffusion in aqueous solution, the concentration of carbon dioxide available to the photosynthetic system approaches very near to that in the environment. This remains true even for the maximum observed rates of photosynthesis and in spite of the relatively slow rate of diffusion of carbon dioxide in water, provided the cells do not exceed a few μ in diameter. With larger algae and the higher water plants, where the whole external surface again represents the area available for entry, the equivalent diffusion path is longer so that even under conditions of moderate rates of photosynthesis there will be a considerable gradient of carbon dioxide between the environment and the reaction centres for photosynthesis. In contrast with land plants thin leaf laminae are common and chloroplasts occur in the epidermal cells with fewer chloroplasts towards the centre of the tissue. The leaf of the land plant shows the development of a different type of diffusion path. The external surface is generally covered by a cuticular layer (which when present in submerged leaves is very thin) and this offers considerable resistance to gaseous diffusion. This layer is, however, broken at intervals by stomata (Gk. *stoma*, a mouth) which give access to a series of air spaces which penetrate throughout the leaf and provide an internal air space with a consequent internal surface many times greater than the external. The resulting structure allows gaseous diffusion (for which the coefficient of diffusion is some 10,000 times greater than for water) up to the wall of the assimilating cell itself. The structure of the land leaf, with its development of an internal system of air spaces, bears an obvious relation to the supply of gases by diffusion. Fig. 2.1 illustrates transverse sections of a submerged and an aerial leaf taken from a single plant of *Sagittaria sagittifolia* and illustrates some of the characteristic differences in structure to which we have referred.

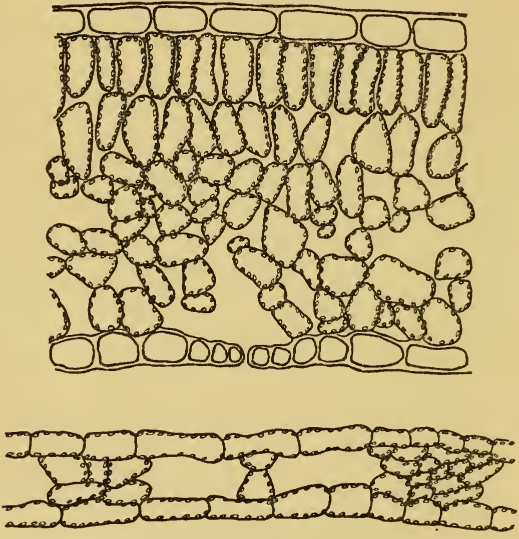


FIG. 2.1. Transverse sections of an aerial (upper) and a completely submerged (lower) leaf of *Sagittaria sagittifolia*. Note the system of air spaces connecting with the external air via the stomata in the aerial leaf; in the submerged leaf the absence of stomata, the presence of chloroplasts in the epidermis and the absence of cuticle.

DIFFUSION THROUGH THE STOMATA

At the end of the nineteenth century it seemed difficult to believe that the stomata provided the main path for the exchange of gases between the external air and the internal atmosphere of the land leaf. Many plants have stomata confined to the lower epidermis of the leaf. Using such a plant, *Nerium oleander*, F. F. Blackman in 1895 showed that the application of petroleum jelly to the lower surface alone was sufficient to markedly decrease the diffusion of carbon dioxide. When the external concentration of carbon dioxide was as high as 6% the absorption by the treated leaf was only one-seventh that by the untreated leaf, demonstrating that the magnitude of cuticular diffusion is small. These observations were extended by Blackman and later by Brown and Escombe to show that in many plants when either only the upper or the lower surface of a leaf was supplied with carbon dioxide the rate of photosynthesis was roughly proportional to the stomatal frequency of the surface supplied. This was not true in all cases, possibly because the stomata were not open to the same extent on both sides. Whilst the lack of precise information as to the degree of stomatal opening in these experiments renders a simple interpretation impossible, nevertheless the results taken as a whole indicate that the main pathway for gaseous diffusion is via the stomata and that diffusion through the cuticle is small by comparison.

TABLE 2.1

THE RATE OF PHOTOSYNTHESIS BY THE TWO SURFACES OF A LEAF
(*from Brown and Escombe* (1900))

	Ratio of stomatal frequency. Upper/lower surface	Rate of photosynthesis. Upper/lower surface supplied with carbon dioxide
Catalpa bignonioides	0 : 100	0 : 100
Nuphar advenum	100 : 0	100 : 0
Colchicum speciosum	100 : 119	100 : 72
Senecio macrophyllus	100 : 126	100 : 82
Rumex alpinus	100 : 269	100 : 137

At first it was found difficult to believe that the stomata which occupy such a small proportion of the total surface area of the leaf represent the main gaseous diffusion path. Brown and Escombe (1905), in a classical investigation, studied the diffusion of gases through multiperforate septa. The septa were made from thin sheets of celluloid and covered the end of a glass tube containing at a fixed distance from the septa sodium hydroxide solution. The septum represented the epidermis of the leaf and the sodium hydroxide the assimilatory mechanism, the whole thus making a simplified physical model of a leaf. With septa 0·09 mm. thick and pores of 0·38 mm. diameter uniformly spaced but varying in number in different septa, they found that diffusion per pore increased as the number of pores diminished, so that it was not until the total pore area had been reduced to a small fraction of the area of the whole septum that the total diffusion was appreciably reduced. For example, with a total pore area of only 1·25% of the septum area, diffusion was still 40% that for the open tube with no septum, whilst a total pore area of 0·31% permitted 14% of the maximum diffusion. These numerical results cannot be directly applied to a consideration of the stomata on a leaf. Their importance lies in the experimental demonstration of the result, independently deduced by Larmor from the laws of diffusion, that provided individual pores are small in diameter and the septum thin a relatively small total pore area will permit a rate of diffusion not far below that of a free surface with no septum.

For a septum of area A, radius R, and thickness l, having n pores equally spaced, each of area a and radius r, Larmor's formula for still air gives the total equivalent diffusion path as $\frac{\pi R}{4}+\frac{A}{na}\left(l+\frac{\pi r}{2}\right)$. This is to be compared with an equivalent diffusion path for the open tube of $\pi R/4$. Even when the pores occupy 1/100th of the total area, i.e. $A/na=100$, provided l and r are small the contribution of the second term is small and the extra resistance due to the septum will be relatively small. For a leaf the term $l+\frac{\pi r}{2}$ is much smaller than in the experiments of Brown and Escombe so that for a given A/na ratio diffusion will be a greater fraction of the maximal value. The effect of wind is to reduce the diffusion path up to the septum, i.e. the term $\pi R/4$, and only to a less extent to alter the extra resistance due to the septum itself. In consequence wind will have a greater proportionate effect on diffusion to a free surface than to or from a multiperforate septum.

Thus Brown and Escombe's experimental work and subsequent studies have established that the relatively impermeable cuticle with its evenly spaced pores of small dimension permits a high rate of diffusion whilst affording considerable protection to the mesophyll cells from external agencies.

THE RATIO OF THE RATE OF PHOTOSYNTHESIS TO THAT OF TRANSPIRATION IN LAND LEAVES

The stomata then are the main pathway for gaseous diffusion, and we next consider the magnitude of the concentration of carbon dioxide in the intercellular spaces of the leaf in terms of the external concentration. The internal concentration will be determined by (1) the diffusion path and (2) the net rate of consumption of carbon dioxide. Heath (1951) and Gabrielson (1948) have both concluded that the concentration of carbon dioxide in the intercellular spaces is about one-third that in the air. An independent method of measuring this concentration is available from measurements of the water vapour lost per gram of carbon dioxide assimilated. For it follows that as the stomata permit the rapid diffusion of carbon dioxide into the leaf they also necessarily result in the loss of water vapour from the leaf. Penman and Schofield (1951) found that during summer growth an acre of turf lost 11,000 tons of water and yielded 130 cwt. of dry matter. The ratio of the rate of transpiration to that of photosynthesis will be under any given set of conditions, as may be seen from equation 2.1, a measure of the ratio of the difference in concentration multiplied by the diffusion constant for water to that for carbon dioxide, because we may assume that the area and equivalent length of the diffusion path between the external air and the intercellular spaces is the same for the two processes.

Thus,

$$\frac{\text{Rate}_{\text{transpiration}}}{\text{Rate}_{\text{photosynthesis}}}=\frac{K_{H_2O}\Delta_{H_2O}}{K_{CO_2}\Delta_{CO_2}} \qquad (2.2)$$

where K represents the respective coefficients of diffusion and Δ the difference in concentration. Measurements of this ratio, i.e. the water lost per carbon gained, indicate that under field conditions the concentration of carbon dioxide in the intercellular spaces may be nearer the external concentration than the measurements of Heath and Gabrielson suggest. (See, for example, the papers of Maskell (1928) and of Penman and Schofield (1951).)

THE DIFFUSION PATH BEYOND THE INTERCELLULAR SPACES

Carbon dioxide diffuses in the gaseous state up to the cell wall; subsequently it must diffuse in an aqueous phase through the mesophyll cell wall and to the point where reaction occurs. This hydrodiffusion path may be regarded as extending to a point at which the concentration of CO_2 would be zero. Although the hydrodiffusion path is only a small distance relative to the air path, since the diffusion of carbon dioxide in water is so much slower than in air, its contribution to the total path is far from negligible. An estimate of its magnitude can be made by comparing under identical conditions the rate of water loss (transpiration) with the rate of photosynthesis. Brown and Escombe calculated these two quantities assuming the diffusion path to be that through the stomata alone (i.e. they had no term corresponding to $\pi R/4$). The calculated rate of transpiration was 6 times greater than the maximum value ever observed and that of photosynthesis 20 times the maximum observed rate. Although their values do not refer to the same environmental conditions we may take it that they indicate a total gaseous diffusion path 6 times that of the stomatal path and an additional resistance to carbon dioxide uptake equivalent to some 14 times the stomatal path or about $2\frac{1}{2}$ times the gaseous diffusion path.

Some control of the diffusion of gases into and out of the leaf is achieved by changes in the aperture of the stomatal pore, the stomata generally tending to close when the plant is placed in the dark or in very dry air. Stomatal closure can of course only affect the stomatal portion of the diffusion path. Since this represents a much greater proportion of the total diffusion path in the case of transpiration, partial closure of the stomata might be expected to result in a greater decrease in the loss of water vapour than in the uptake of carbon dioxide. Such a differentiation between the two processes has been observed by a number of investigators. When the stomata are completely closed there remains the exchange of water and carbon dioxide through the cuticle. The cuticle is a complex of fatty acids, alcohols, and hydrocarbons. It has not yet been shown whether its chemical nature favours the passage of carbon dioxide against that of water (as, for example, would a polythene layer).

DETAILED STRUCTURE OF THE ASSIMILATORY CELL AND CHLOROPLAST

In the mesophyll cell the cytoplasm and its inclusions must be regarded as the seat of photosynthesis. The chloroplasts are clearly defined structures within the cytoplasm and the photosynthetic pigments are confined to them. When removed from the cell and seen in a compact mass they appear almost black.

The chloroplasts of the leaf cells are ellipsoidal or have an unsymmetrical lens shape. They vary in size, the longest axis often being between 3 and 5 μ although it may reach 10 μ or be as small as 2 μ. The number of chloroplasts per cell varies from several hundred down to only a few, in general there being a greater number in the cells of the palisade than in those of the spongy mesophyll. In the algae frequently each cell contains only one chloroplast, the shape of which varies, e.g. the stellate chloroplasts of *Zygnema*, the cup-shape in *Chlorella*, and the long spiral of *Spirogyra*.

Chloroplasts usually appear to consist of a membrane containing a ‘stroma’ in which ‘grana’ are distributed. It is thought that the photosynthetic pigments are largely confined to the grana. The number of grana in a single chloroplast may vary from about 10 to several hundred. Studies with the electron microscope have shown individual grana as disc-like bodies consisting of lamina at right angles to the short axis. The lamina vary in diameter but are commonly about 0·6 μ in diameter corresponding to the diameter of the granum (Granick (1949)).

In the blue-green algae and the photosynthetic bacteria no chloroplasts are visible. It is, however, possible to make preparations which show that the pigments are present in small particles some 0·5 to 0·3 μ in diameter, too small to be visible with the light microscope. With the electron microscope the particles appear as disc-like structures similar to the grana of other plants (Pardee, Schachman, and Stanier (1952)).

Very thin sections of *Euglena* have shown under the electron microscope the chloroplast as consisting of about 20 continuous layers with no sharp differentiation into individual grana. In this case it appears that the chloroplast is equivalent to a single granum.

Thus evidence is accumulating that the chloroplast is a definite structure consisting of differentiated layers. How the constituent pigments are related to these layers has not yet been determined experimentally. Evidence is accumulating that the submicroscopic structure of the pigment complex may be an important factor in a physico-chemical interpretation of photosynthesis.

The plastids show many points of similarity to mitochondria, but it is not certain that these two cytoplasmic structures are analogous. The chloroplasts probably possess a variety of synthetic mechanisms in addition to those directly concerned with photosynthesis.

Evidence as to whether the whole of the photosynthetic mechanism is located in the chloroplasts is still lacking. Some suggestion that this is not the case was obtained from experiments with radioactive carbon (Frenkel, 1941). In the dark, carbon fixation occurred almost exclusively in the cytoplasm whereas during illumination four-fifths of the activity was found in the chloroplasts. The processes involving carbon dioxide in photosynthesis may therefore be associated with the cytoplasm, and this would involve diffusion of intermediates as one step in the over-all mechanism.

Chloroplasts do not arise *de novo* but are independent self-reproducing entities, inherited generally from the cytoplasm of the female cell. Thus Ternetz found with a species of *Euglena* that it would give rise to cells without chloroplasts or leucoplasts when grown in the dark; these cells when grown separately never formed chloroplasts again. The development of the chloroplast is determined by Mendelian factors and by factors depending on the functional and physiological differentiation of the tissues. For example, in maize numerous independent genes have been identified which determine albinism and influence the pigmentation of the chloroplasts.

CHAPTER 3

THE CHEMISTRY OF PIGMENTS ASSOCIATED WITH THE CHLOROPLAST

The pigments of the chloroplast belong to two chemical classes; tetrapyrrolic compounds, to which class chlorophyll belongs, and the carotenoids which include the yellow xanthophylls and the orange carotenes. The colour of leaves may be modified by the presence of other pigments, the red or purple anthocyanins, but these are dissolved in the cell sap and are absent from the cytoplasm. The anthocyanins play no direct part in photosynthesis. In the red algae additional pigments which often mask the green colour of the chlorophyll are present and intimately associated with the chloroplasts. These are chemically related to the chlorophylls, and are tetrapyrrolic compounds. The light absorbed by them can be used in photosynthesis.

THE CHLOROPHYLLS

The chlorophylls are readily extracted from fresh green tissues of plants by acetone and from powdered dried leaves by both acetone and alcohol. It is necessary in the latter case to add water to the extent of 10 to 20% or extraction is incomplete. The pigments may be separated by differential distribution between immiscible solvents or by fractional absorption. Willstätter and Stoll (1913) should be consulted for details of this and other procedures. The first method, used by Willstätter, proceeds by adding the acetone extract to an equal volume of petrol ether and a little water. The petrol ether separates containing almost all the chlorophyll. Most of the xanthophyll and some chlorophyll *b* is removed by shaking with aqueous methanol. By repeated washing with water the petrol ether solution loses its fluorescence and the chlorophyll begins to come out of solution. It can then be obtained by filtering through a thin layer of talc and the

TABLE 3.1

TABLE OF PIGMENTS

			Maximum absorption	
Chlorophylls				
Chlorophyll *a*	All green plants	Tetrapyrrolic plus phytol. Fifth ring with 5C atoms. IVth pyrrole with 2H. Mg atom	Red and blue-violet	Probable protein-pigment complex. Part hydrophobic, part hydrophillic
b	Not brown, red algae, diatoms blue-green			
c	Brown algae, diatoms			
d	Red algae			
Protochlorophyll	Etiolated plants	No 2H in pyrrole rings	Near red and blue-violet	
Bacteriochlorophyll	Purple S bacteria	IVth and II pyrrole + 2H	Infra-red and blue-violet	
Bacterioviridin	Green S bacteria	Probably similar to chlorophyll *a*	Red and blue-violet	
Phycobilins				
Phycocyanin	Blue greens, also red algae	Tetrapyrrolic open chain. No Metal	Orange red	Protein-pigment compound. Water soluble
Phycoerythrin	Red algae, blue-greens	do.	Green	
Carotenoids	Variable in different plants	Long chain unsaturated hydrocarbons	Blue and blue-green	Fat soluble
Xanthophyll (=Carotenols)		Contains 1 or more O atoms		
Carotenes		Do not contain O atoms		

carotene removed from this by repeated washing with petrol ether. A mixture of two chlorophylls, chlorophyll *a* and chlorophyll *b*, now remains, and these can be separated since chlorophyll *a* is the more soluble in petrol ether whilst *b* is the more soluble in methyl alcohol. Many fractionations are necessary to remove chlorophyll *a* from chlorophyll *b*. The second method of separation was devised by Tswett. If the petrol ether extract is dehydrated and then filtered through a column of powdered sucrose or calcium carbonate the various pigments are adsorbed in different bands along the column. The major bands are easily identified by their colours, the blue-green of chlorophyll *a*, the neutral green of chlorophyll *b*, the various yellow xanthophyll bands, and the yellow carotene which is not adsorbed on the column but comes through in solution. The best method of isolation

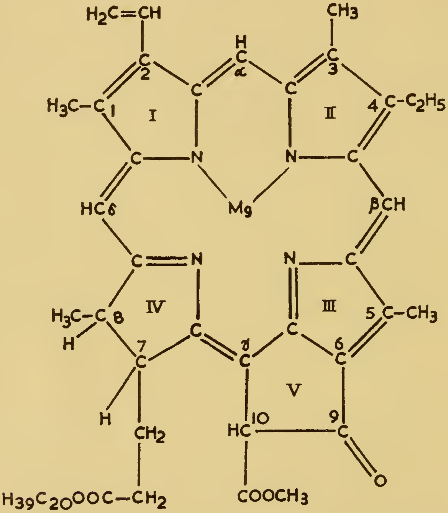
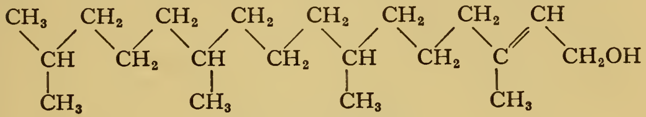


FIG. 3.1. Structural formula of chlorophyll *a*. Note the methyl group attached to carbon atom 3 which becomes a formyl group in chlorophyll *b*; the phytol group attached to the chain at carbon atom 7; the cyclopentanone ring V.

combines the two procedures: a partial purification by partition with solvents followed by adsorption on a suitable column (Rothemund, 1944). When obtained in pure form both chlorophylls are nearly black, wax-like solids which do not show any tendency to crystallize in bulk.

The chlorophylls (Fig. 3.1) are methyl phytol esters of the parent dicarboxylic acids, the chlorophyllins. Phytol ($C_{20}H_{39}OH$) is a long chain alcohol, containing one double bond, which is related to the carotenoids and can be regarded as derivable from vitamin A by hydrogenation.



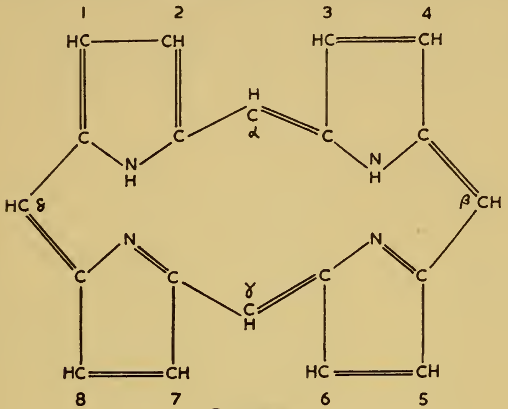
Phytol ($C_{20}H_{39}OH$)

The enzyme chlorophyllase hydrolyses the phytol group from chlorophyll. In acetone the resulting green product is chlorophyllin, whilst in ethanol or methanol the phytyl is replaced by ethyl or methyl to give ethyl or methyl chlorophyllide. These esters crystallize easily and were the first chlorophyll derivatives to be obtained in crystalline form. With excess phytol chlorophyll is synthesized from chlorophyllin in the presence of the enzyme, i.e. the reaction is reversible. Chlorophyllase has not been obtained in a soluble form; it is present in the insoluble residue of the chloroplast fraction after acetone treatment; it has also been found in roots. Some plants show little chlorophyllase activity, e.g. *Urtica* and the grasses; others show good activity, e.g. *Datura*, *Heracleum*, and *Galeopsis*. It follows that in the preparation of chlorophyll it is desirable to use plants which contain little chlorophyllase.

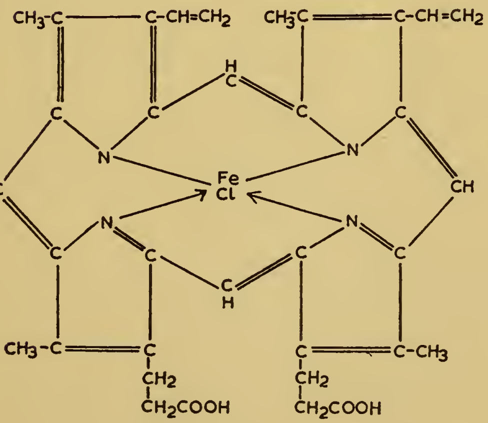
The chlorophylls are remarkable in being a group of natural substances in which magnesium is present in a nonionic form. Treatment with dilute acid results in the solution in alcohol becoming brown and a dark precipitate of the corresponding magnesium-free compound, the phaeophytin, is obtained. In organic solvents containing water under neutral conditions chlorophyll cannot be reformed even with excess of magnesium salts; it is however slowly resynthesized in absence of water by heating phaeophytin with a modified Grignard reagent, e.g. ethoxy magnesium bromide. Ordinary Grignard reagents react immediately at ordinary temperatures to give the magnesium derivative, but in this case the side chains are also attacked. In contrast with its inertness towards magnesium ions, phaeophytin readily reacts with other metallic salts, e.g. copper salts. Hence when carrying out experiments with chlorophyll derivatives copper and other metals must be rigorously excluded. The copper derivative of phaeophytin is green, stable to light, and not affected by acids; the zinc derivative is like chlorophyll in that acid readily removes the zinc; the iron derivative is dark green and shows some likeness to the iron porphyrin compounds which are the prosthetic groups of respiratory pigments.

The atom of magnesium is situated in the centre of the molecule and is surrounded by four pyrrole nuclei, and thus chlorophyll has a similar structure to haemin, the latter however containing a central iron atom (Fig. 3.2). In general plan phaeophytin and phaeophorbide (the result of removing magnesium from a chlorophyllide) are similar to the porphyrins in structure but differ in the addition of two more hydrogen atoms to the pyrrol system at C atoms 7 and 8. This alters a sequence of conjugated double bonds, which in the porphyrins is part of the ring system, and results in a marked change in the type of absorption spectrum, the chlorophyll derivatives absorbing much more strongly at longer wavelengths than the corresponding porphyrin derivatives. The simpler chlorins, derived from chlorophyll derivatives by pyrolysis, are readily oxidized to the corresponding porphyrins. The chlorins derived from chlorophyll *b* (i.e. with a formyl group at carbon atom 3) are sometimes referred to as rhodins. The introduction of the two hydrogen atoms which occurs in the pyrrol ring IV of the porphyrin results in the appearance of two asymmetric carbon atoms, and in agreement with this is the fact that not only chlorophyll, but also the pheophorbides and simpler derivatives of the parent 'chlorin' show optical activity.

When a fresh preparation of chlorophyll is saponified with strong methanolic alkali an immediate change of colour occurs, the green changing to a yellowish brown (if a



PORPHIN



PROTOHAEMIN

FIG. 3.2. Structural formulae of porphin and protohaemin.

mixture of chlorophylls *a* and *b* is used); after a short time the original green colour returns. This reaction, the 'phase test', is not shown by alcoholic solutions of chlorophyll which have been stored. Willstätter referred to the alteration of chlorophyll due to storing as 'allomerization', and it was later shown by Conant and by Fischer that this was due to oxidation of the hydrogen atom in the cyclopentane ring at

TABLE 3.2

NOMENCLATURE OF PIGMENTS

Ethyl chlorophyllide:	Chlorophyll with magnesium but ethanol replacing phytol
Phaeophytin:	Chlorophyll minus magnesium
Phaeophorbide:	Chlorophyll minus magnesium and phytol
Phaeoporphyrin:	Class of porphyrins corresponding to chlorophyll: they contain two hydrogen atoms less than chlorophyll but still contain the extra 5C ring which is lacking in porphyrins derived from haem
Chlorin:	Chlorophyll minus magnesium, phytol, and methanol and produced from phaeophytins by action of alkali: the 5C ring is opened. They contain two hydrogen atoms more than porphyrins
Porphyrin:	Metal free tetrapyrrolic compounds derived from haem. Also by vigorous degradation of chlorophyll derivatives
Porphin:	Basic metal free tetrapyrrolic structure with hydrogen attached to all carbon atoms 1 to 8

C10 by atmospheric oxygen. The 'phase test' is shown by compounds containing an intact cyclopentane ring. Thus phaeophytin and protochlorophyll (see later) also give the phase test. During saponification the cyclopentane ring is opened by hydrolysis, thus producing a chlorin which in the absence of oxygen can be converted back again to the original compound with a closed ring. In the presence of oxygen the side chains undergo oxidation and give a variety of chlorins.

Properties of chlorophyll in solution

1. *Absorption of light.* On a molecular basis the absorption of light by chlorophyll is very high, being for example three times greater than that of the chemically related pigment, haemoglobin, in the visible region. In solution both chlorophylls show absorption spectra of a similar type characterized by a very strong band in the red and one in the violet. In ether chlorophyll *a* appears green-blue, while chlorophyll *b* is a more yellowish green. In a thick layer (or in high concentration) solutions of both chlorophylls transmit only red light. At the red end of the spectrum the maximum

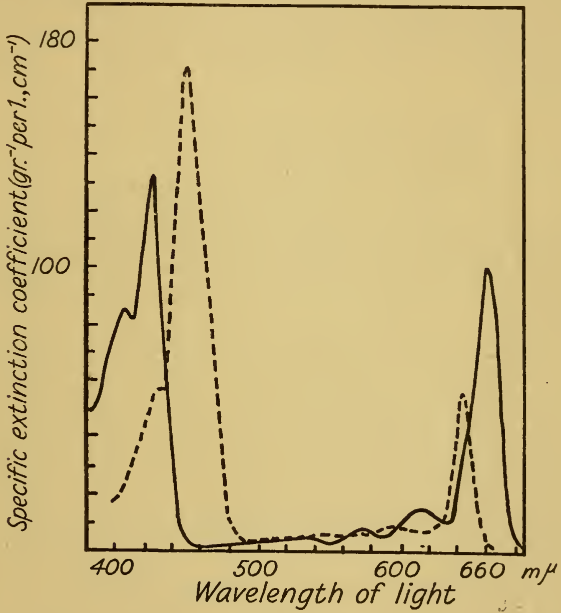


FIG. 3.3. Absorption curves of chlorophyll *a* (———) and chlorophyll *b* (– – – –) in ethyl ether. (Data of Zscheile and Comar.)

of the chlorophyll *a* absorption band lies at a longer wavelength than that of chlorophyll *b*, and conversely at the violet end (Fig. 3.3). Hence by measurements of the absorption of light at two specified wavelengths in an acetone extract of green leaves the quantities of both chlorophylls *a* and *b* may be determined (Mackinney, 1941). If D_{663} and D_{645} are the optical densities at the two wavelengths 663 mμ and 645 mμ then these are related to the concentrations of chlorophyll *a*, c_a and chlorophyll *b*, c_b by the following equations:

$$D_{663}=82{\cdot}04c_a+9{\cdot}27c_b$$

$$D_{645}=16{\cdot}75c_a+45{\cdot}6c_b$$

or

$$C=c_a+c_b=0{\cdot}0202D_{645}+0{\cdot}00802D_{663}$$

Both the extinction coefficient and the position of the band maxima vary with the solvent used, and small amounts of impurity in the solvent may appreciably alter the absorption curves.

2. *Fluorescence.* In organic solvents both chlorophylls show a characteristic fluorescence spectrum consisting of a strong band in the red and a weaker band in the far red. The intensity of the fluorescence of a solution in acetone or ether represents about 10% of the light energy absorbed. Just as with the absorption curves the position of the bands and the intensity of fluorescence are different in different solvents. Thus Livingston found that solutions of ethyl chlorophyllide in pure dry benzene showed very little fluorescence, the intensity being one-twentieth that in methanol or ether. If a little water, methanol, or ether is added to the benzene the fluorescence is now even greater than that in pure methanol. The second solvent need be added, however, only in an amount equal on a molecular basis to the amount of chlorophyll present and thus it has been suggested that the fluorescence of chlorophyll is due to the formation of a chlorophyll complex formed with the added substance. The fluorescence of solutions of chlorophyll can be 'quenched' by the addition of such substances as quinone and by other oxidizing agents, and also by phenyl hydrazine (a very low

concentration of phenyl hydrazine actually stimulates fluorescence of chlorophyll in benzene). The fluorescence is also quenched by oxygen to an extent of about 20% in air. Removal of the magnesium to form the phaeophytin has little effect on the fluorescence but replacement of the magnesium by copper abolishes the fluorescence. The porphyrin series is similar, both free porphyrins and the magnesium derivatives being fluorescent whilst the copper derivatives are not.

3. *Chemiluminescence.* The luminescence of tetrapyrrolic compounds when heated in organic solutions was first discovered with the compound magnesium phthalocyanine. Free porphyrins or metallic derivatives other than the magnesium compounds, with the exception of zinc which shows a weaker effect, do not luminesce. Thus the ability of a pigment to luminesce does not simply depend on the capacity for fluorescence but in the case of these tetrapyrrolic compounds rather on the presence of magnesium. A solution of chlorophyll in tetralin (1 : 2 : 3 : 4-tetrahydronaphthalene) shows a strong orange-red glow when the temperature is raised to about 200° C., the spectrum of the emitted light resembling the fluorescence spectrum of chlorophyll. The maximum is, however, shifted nearer to the violet due in the first place to the thermal degradation of the side groups of the chlorophyll molecule. Other organic liquids can be used in place of tetralin, e.g. higher paraffins, natural oils or fats.

4. *Photochemical sensitizations.* As early as 1874 Becquerel showed that chlorophyll could be used as a sensitizer for the photographic plate and since that time a variety of reactions photocatalysed by chlorophyll have been described. The reactions fall into two classes: (*a*) oxidations involving molecular oxygen and (*b*) oxido-reductions involving hydrogen transfer between two reactants.

In the first class Gaffron (1927) showed that chlorophyll photocatalysed the oxidation of allyl thiourea in acetone or of thiourea in pyridine. The efficiency decreases as the concentration of thiourea is decreased or the concentration of chlorophyll increased. For a certain range of light intensities one molecule of oxygen is consumed under the best conditions for each light quantum absorbed by the system; thus in recent years Warburg has used this reaction as a light meter. Protoporphyrin will also sensitize the reaction although it may be noted that unlike its magnesium compound or chlorophyll it does not luminesce when heated in organic solutions. Porphyrins if present in the blood are well known to sensitize animals to light, and this may even result in the death of the organism. *In vitro*, the addition of porphyrin causes the serum of an animal to take up oxygen in the light. The light dependent oxidation of certain compounds in organic solvents gives rise to peroxides; isoamylamine, for example, gives an amine peroxide when illuminated with chlorophyll in presence of oxygen.

In the second class, the photosensitized oxidoreductions, the best known reaction is the reduction of methyl red by phenylhydrazine. In reactions involving photosensitization by dyes in which the dye substance is itself a reactant it is generally found that absorption of light increases the tendency to accept hydrogen. When the reaction is photosensitized by the presence of another pigment, as for example chlorophyll, it may not be necessary to assume that the photosensitizer directly takes part in the transfer of hydrogen. It may simply replace the direct absorption of light by the reactant. The problem as to how the energy is then transmitted to the reactants, especially when complex organic molecules are involved, has not yet been solved.

In autoxidation reactions such as that of thiourea no observable quenching of the chlorophyll fluorescence is observed. On the other hand in the oxidoreduction reaction both methyl red and phenyl hydrazine independently quench the fluorescence of chlorophyll.

5. *Reversible bleaching of chlorophyll solutions*. When solutions of chlorophyll or chlorophyllides are strongly illuminated in an organic solvent in the absence of oxygen Porrett and Rabinowitch (1937) showed that the absorption coefficient is reversibly lowered by a small amount. The effect is far too small to be observed as a fading of the colour. Oxygen

in a concentration as low as 10^{-6} M. entirely prevents the bleaching. Carbon dioxide has little effect, whilst formic acid greatly increases it. So far no reversible changes in the spectrum of chlorophyll under biological conditions have been observed. Thus there is no evidence yet as to whether reversal changes in the pigment observed in solution have any bearing upon the function of chlorophyll in the cell.

6. *Irreversible bleaching*. Solutions of chlorophyll also bleach irreversibly in the light. In chloroplast preparations and in the living cell the pigment appears to be protected in some way and is only bleached under extreme conditions.

Distribution of chlorophylls

The content of chlorophyll in green leaves varies between about 0·05 to 0·20% fresh weight, generally consisting of about 2·5 parts of chlorophyll *a* to one part of chlorophyll *b*. Leaves of yellow-green varieties may have a far greater relative amount of chlorophyll *a*, the total amount of chlorophyll often being less than one-third that in green varieties. A greater proportion of chlorophyll *a* is frequently found in 'shade' plants compared to 'sun' plants.

Great variation in chlorophyll content occurs in the different classes of algae. In *Chlorella* the chlorophyll content may be as much as three times that in leaves (see Appendix I), which means that the concentration in the chloroplasts is of the order of 0·05M. and perhaps therefore even 0·2M. in the grana. In *Chlorella* the ratio of chlorophyll *a* to *b* may be as great as 3 : 1, whereas in many other green algae it is only 1·4 : 1. Chlorophyll *b* is absent from the brown algae, red algae, diatoms and the blue-green algae. Thus chlorophyll *a* is the most abundant in all plants and with the exception of the bacteria is common to all types of autotrophic plants. In the brown algae, diatoms, and the flagellates, from which chlorophyll *b* is absent, a different pigment chlorophyll *c* is present in an amount about one-tenth the total chlorophyll content. In the red algae chlorophyll *a* is accompanied by chlorophyll *d*, the main absorption band of which is further in the red (6,860 Å in ethyl ether).

Etiolated seedlings showing a yellow colour due to carotenoid pigments contain also the pigment protochlorophyll which is converted to chlorophyll in the light. Protochlorophyll is more oxidized than chlorophyll, the two hydrogen atoms in positions 7 and 8 being absent.

Bacteriochlorophyll is the green pigment present in the purple photosynthetic bacteria. It is similar to chlorophyll *a* but has the vinyl group $—CH{=}CH_2$ oxidized to $—CO—CH_3$ in position 2 and double bonds in two of the pyrrole rings hydrogenated, not one as in chlorophyll. The main absorption band is in the infra-red (7,700 Å in methanol) with another band at about 6,000 Å. The green photosynthetic bacteria contain the pigment bacterioviridin, whose structure may be intermediate between that of chlorophyll *a* and bacteriochlorophyll. The absorption spectrum is similar to that of chlorophyll *a*.

THE CAROTENOIDS

Carotenoids occur in all green plants in the plastids, but additional members of this group of compounds are also found in the non-green parts of plants, such as the perianth and brightly coloured fruits, and also widely throughout the animal kingdom. A carotenoid of much importance in photochemical reactions in animals is visual purple. The original identification of two carotenoid compounds in green leaves, xanthophyll and carotene, has now been extended to the chromatographic recognition of a number of different isomeric forms. A brief list of carotenoids with their distribution is given in Table 3.3. It is by no means complete. Chromatographic analysis has revealed the presence, in, for example, the higher plants, of a wide variety of members of the class of xanthophylls, in some cases the relative amount of some minor constituent being shown to be controlled by a genetic factor. In the algae taxonomic groups may be defined by the presence of a particular carotenoid pigment. Carotenoid pigments have been found in mitochondria in both plants and animals, and it is possible that a minute quantity of carotenoid pigment is universal in the cytoplasm of aerobic organisms.

TABLE 3.3

DISTRIBUTION OF CAROTENOID PIGMENTS

Plant	Main constituents	Other components
Higher plants, ferns, mosses, green algae	β-Carotene Luteol	Violaxanthol Neoxanthol Zeaxanthol α-Carotene
Siphonales	α-Carotene (differs from other Chlorophyceae)	
Red algae	β-Carotene	Luteol α-Carotene
Brown algae, diatoms	β-Carotene	Fucoxanthol
Blue-green algae	β-Carotene	Myxoxanthol
Photosynthetic anaerobes	Spirilloxanthin	Rhodopol Flavorhodene Rhodopurpurene

Note: In the above table the nomenclature giving a precise chemical indication of the groups present has been given. Thus the ending -ol indicates the presence of hydroxy compounds and luteol, zeaxanthol are preferred to lutein, zeaxanthin, &c.

Preparation of the leaf carotenoid pigments

An alcohol or acetone extract of leaves, prepared as described earlier, contains all the pigments of the chloroplast. The carotenoids are stable to alkali, so that after saponification, as in the phase test, the carotenoids remain in the ether phase whilst the green pigments pass into the aqueous phase. The pigments are transferred to petrol ether and 92% aqueous methyl alcohol added. The carotenes (hydrocarbons) remain in the petrol ether whilst the xanthophylls (having hydroxyl groups) pass into the alcoholic phase. This was the basis of the method used by Willstätter and Stoll. Separation can as previously mentioned be obtained by using a chromatographic technique and later work with this method has shown the large number of carotenoid pigments present in the leaf.

Ratio of carotenoids to chlorophyll

The molecular ratio of the carotenoid to chlorophyll present in leaves is very variable. In normal green leaves there are more molecules of chlorophyll than carotenoid, in shade leaves the ratio sometimes approaching 5 : 1. In etiolated plants the carotenoids are present before greening has occurred, although they may increase in amount concurrently with the chlorophyll.

Chemical properties

The carotenoids contain a large number of double bonds, many of which form a conjugated system. This gives rise to the possibility of a large number of cis-trans isomers, a number of which have been isolated.

In leaves the main carotenoid constituent is β-carotene, a member of the class of hydrocarbons the carotenes, $C_{40}H_{56}$. It crystallizes easily in steel-blue crystals, which are deep red by transmitted light. In most organic solvents it forms a yellow solution but in carbon disulphide it forms an orange solution. Both the pure solid and the solutions are oxidized on exposure to air, giving amongst the products β-ionone. In common with all other carotenoids carotene gives a deep blue colour with concentrated sulphuric acid. The xanthophylls constitute the class of carotenoids which contain oxygen. The most common compound of this type in green leaves is luteol, a dihydroxy-carotene, $C_{40}H_{56}O_2$. During autumn, when changes occur in the carotenoids of the leaf an isomer of luteol, zeaxanthol, becomes the most abundant constituent.

Like most unsaturated compounds the carotenoids are very reactive and are easily oxidized by molecular oxygen. However, in the living cell the carotenoids appear to be stable, although on breaking the cell rapid oxidation sometimes takes place. The oxidative breakdown is less under conditions where enzymes are destroyed and this has led to the suggestion that the cell contains enzymes catalysing the oxidation of carotenoids. It has also been suggested—particularly in certain algae—that some of the carotenoid pigments are combined with protein in the cell. The brown algae turn green on heating owing to an alteration in the state of the fucoxanthol. It is clear that many aspects of carotenoid biochemistry remain to be explored.

The absorption of light by carotenoids

All carotenoids strongly absorb light in the violet end of the visible spectrum, showing two or three separate bands (Fig. 3.4). The absorption extends into the blue to different extents according to the structure of the compound. The carotenoids of the bacteria absorb further to the red than those of higher plants. Even more than in the case of the

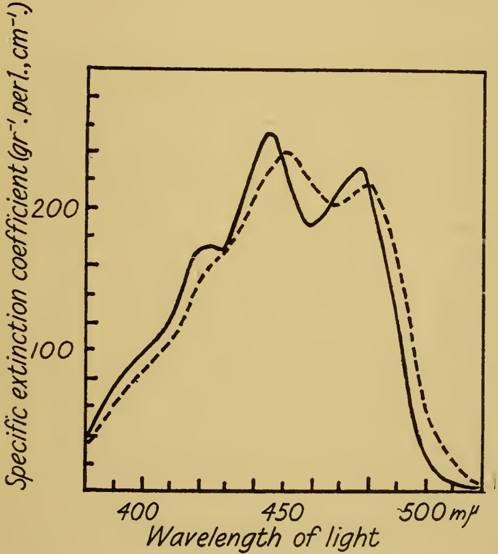


FIG. 3.4. Absorption curve for typical carotenoids; luteol (———), zeaxanthol (— — — —) dissolved in ethanol. (Data of Zscheile *et al.*)

chlorophylls the position of the bands varies according to the solvent used.

PHYCOERYTHRIN AND PHYCOCYANIN

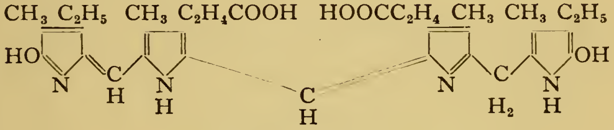
These pigments are associated with the photosynthetic mechanism of the red and the blue-green algae; in the latter phycocyanin predominates. They differ completely from the chlorophylls and the carotenoids in being soluble in dilute aqueous salt solutions, the prosthetic groups being combined with proteins of the globulin class.

Preparation

Phycoerythrin and phycocyanin are obtained directly when algal cells are broken in dilute aqueous salt solutions. Alternatively a little chloroform may be added to the algae in sea water when the pigments diffuse out, leaving the other plastid pigments in the cell. One of the best sources for preparation is the large red alga *Porphyra tenera*. After extraction the pigments are separated by fractional precipitation with ammonium sulphate and can be prepared in crystalline form. From a purified mixture the two components will crystallize independently, giving simultaneously brilliant red and blue crystals of phycoerythrin and phycocyanin respectively. When solutions of the pigments are dialysed free from salt they precipitate and in this form are easily denatured and undergo a change in colour. In crystalline form and in the presence of 10% ammonium sulphate, however, Lemberg has stored preparations unchanged for two years (Lemberg and Legge, 1949). Phycoerythrin and phycocyanin are to be regarded as two classes of chromoproteins; differences which depend on the plant source are known to occur and may be due to the protein part rather than to the prosthetic group.

Chemistry of the prosthetic groups

The molecular weights of phycoerythrin and phycocyanin were determined with the ultracentrifuge and found to be 290,000 and 273,000 respectively. The pigment groups are firmly bound to protein and require strong acid hydrolysis to separate them. Once separated they may be esterified and purified by extraction with chloroform. The prosthetic groups were first isolated by Lemberg, who was able to prove their relationship to the bile pigments. With phycoerythrin he identified the bile pigment meso-bilierythrin and with phycocyanin meso-biliviolin. Both pigments are tetrapyrrolic derivatives with the pyrrol rings in open chain. The erythrins and the violins appear to be isomeric structures, but there is still some doubt concerning the structure of the bilierythrin. The two compounds are readily interconvertible and both give on oxidation meso-biliverdin, the structure of which is well established. The prefix meso-indicates that the two side chains corresponding to the vinyl group in positions 2 and 4 in haem are converted into ethyl groups.


CH_3 C_2H_5 CH_3 C_2H_4COOH $HOOCC_2H_4$ CH_3 CH_3 C_2H_5
HO
N
C
H
N
H
C
H
N
C
H_2
N
H
OH

meso-biliviolin (Lemberg)

The pigments are presumed to be attached through the carboxyl group to a peptide link of the protein, probably by a twofold attachment. Each molecule of chromoprotein contains at least 8 molecules of prosthetic group (the value obtained by chemical analysis of the yield of isolated prosthetic groups) or perhaps as many as 16 (from comparison of spectral absorption coefficients). If the latter estimate is correct the ratio of prosthetic groups to protein weight is similar to that in haemoglobin (4 haems per 68,000) or in cytochrome *c* (1 haem per 14,000).

Optical properties

Phycoerythrin absorbs in the visible mainly in the yellow region of the spectrum and shows three maxima. Phycocyanin absorbs somewhat further into the red (Fig. 3.5). In dilute salt solutions phycoerythrin shows an orange fluorescence whilst phycocyanin shows a red fluorescence. In both cases the fluorescence appears stronger than that of chlorophyll in organic solutions, but this may be because the colour of the fluorescence is nearer to the maximum colour sensitivity of the eye. There appear to be no measurements of the fluorescent yield. The fluorescence of the pigments is completely lost by denaturation of the protein component, even under conditions where precipitation does not take place. (This may be compared to the loss of fluorescence of chlorophyll in the chloroplasts under mild conditions—such as heating to 55° C.—which may also be presumed as due to denaturation of a protein-pigment complex.) With the denatured pigments the fluorescence may be restored by combining the prosthetic groups with zinc. Indeed the spectra of the chromoproteins seem to resemble those of the zinc compounds rather than that of the free prosthetic groups; yet, so far, the purified chromoproteins have been found to be metal free.

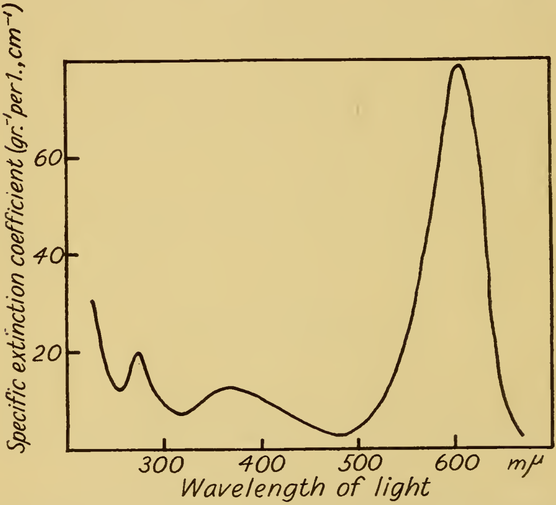


FIG. 3.5. Absorption curve for phycocyanin. (Data of Svedberg and Katsurai.)

HAEMATIN COMPOUNDS PRESENT IN GREEN LEAVES

In addition to the pigments concerned in the absorption of light, another group of porphyrin compounds present in the leaf is the haematins. Although present in molar concentrations less than one-hundredth that of chlorophyll, in the leaf the haem pigments are in a greater concentration relative to the respiratory activity than in other organisms.

In addition to catalase, peroxidase, and the cytochromes which occur throughout the plant (see Table 3.4), the leaves of higher plants also contain two cytochrome components believed to be characteristic of plant tissue. Acetone treatment removes the chlorophylls and carotenoid pigments but does not result in appreciable loss of the haematin compounds. Direct spectroscopic examination of the acetone-treated leaf preparation showed the presence of a non-autoxidizable cytochrome. Fractionation of the pressed juice from macerated leaves using ammonium sulphate yielded both a non-autoxidizable and an autoxidizable cytochrome component. The autoxidizable component had in the reduced form an α band at 559·7 mμ and was called cytochrome b_3 on account of some resemblances to cytochrome b_2 (α band 556·3 mμ). The second component cytochrome *f* could not be extracted in an unmodified form from acetone preparations from leaves, but was obtained from fresh leaves ground with ethanol containing ammonia. Cold acetone was added to the alcoholic solution when part of the *f* component could be obtained from the precipitate in aqueous solution (Davenport and Hill, 1951). Cytochrome *f* is non-autoxidizable and gives no reaction with carbon monoxide. Its potential is more oxidizing than that of cytochrome *c* by about 0·1 volt. The absorption spectrum of ferrocytochrome *f* is similar to that of cytochrome *c*, but the bands are more sharply defined and lie 5 mμ nearer the red end of the spectrum. The α band is asymmetric and in solutions more alkaline than pH 9·0 it can be observed to split into two components, a strong narrow band at 556 mμ and a narrow and weaker band at 551 mμ; the effect is reversible up to pH 12, above which denaturation of the protein occurs. The prosthetic group and its mode of attachment is the same in cytochromes *c* and *f* and the observed differences presumably arise from differences in the protein component.

Cytochrome b_3 is not confined to green organs; cytochrome *f* appears to be confined to the chloroplasts. Cytochrome *f* predominates in the leaf, its concentration being about 10 times greater than that of the other cytochromes. The cytochromes show high specificity in oxidation-reduction reactions. In a later chapter we shall see that photosynthesis is essentially an oxidation-reduction reaction and it is possible that cytochrome *f* may play the part of a carrier in the process. It may be significant that the ratio of the rate

TABLE 3.4

HAEMATIN COMPOUNDS IN THE PLANT

Compound	Location	Identification
Peroxidase	All over plant	Characteristic reaction; spectrum of concentrated protein fraction
Catalase	Chloroplast and rest of plant	Characteristic reaction. Isolated in one case
Cytochrome a a_3	Throughout plant but in highest concentration in meristematic tissues	Inferred from cytochrome oxidase activity
b		Inferred from succinic oxidase activity
b_3		Spectrum; isolation in impure form
c		Spectrum; isolated
Cytochrome f	Only in green tissues	Spectrum and oxidation reduction properties. Isolated in impure form

of respiration/cytochrome *c* in yeast approximates to the ratio of rate of photosynthesis/cytochrome *f* in leaves, both rates being expressed as Qo_2.

THE STATE OF THE PIGMENTS IN THE PLASTID

The phycobilins are as we have seen water soluble chromo-proteins whilst chlorophyll and the carotenoids are soluble only in organic solvents. Thus we are led to postulate at least a two-phase system within the chloroplast. In gross composition the plastids are 50% water, 25% protein, 15% lipoid, and about 10% pigment. As to the detailed chemical structure and organization of the plastid there is little direct evidence.

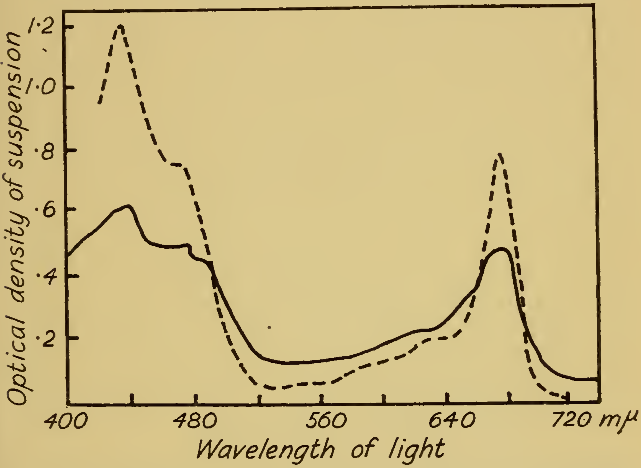


FIG. 3.6. Comparison of the absorption spectrum of a suspension of *Chlorella* cells (————) and that from the combination of the spectra of the isolated pigments in solution (— — — —). (After Emerson and Lewis.) Maxima for absorption in the red made to coincide.

The association of the pigments with other constituents of the plastid is shown by the difference in the position of the maxima of the absorption bands of the pigments in the plant cell compared with that of the pigments in organic solution.

Subsequent to extraction from the cell both the absorption and the fluorescence bands shift towards shorter wavelengths, e.g. the chlorophyll red absorption band maximum changes from about 680 mμ to 660 mμ, whilst in bacteriochlorophyll it is shifted, possibly by 100 mμ, from the far to the near infra-red (there still remaining considerable uncertainty as to the correlation between the bands of the pigment in extract and those of cell suspensions). The absorption maxima of the carotenoids in extract are shifted towards shorter wavelengths by some 10–30 mμ. All these figures are rough estimates derived from an attempt to analyse the absorption curve of a living cell suspension into a number of component curves. There remains considerable uncertainty in any such analysis since, in addition to the shift in the absorption maxima, the bands of the pigments in the cell also appear broader owing, in part, to the effect of scattering (see Fig. 3.6).

Green aqueous juices may be extracted from green leaves either by pressing or by grinding at low temperature in distilled water. The absorption spectrum of the juice is practically identical with that of the leaf and the chlorophyll is stable towards oxygen and light in the same way as that in the plastids. Such preparations show only a weak fluorescence. Similarly bacteria may be ground or broken, using ultrasonic waves and a clear colloidal solution obtained with an absorption spectrum similar to that of bacterial cell suspensions. These preparations are irreversibly altered by heat, suggesting that the properties shown depend on the presence of native protein. Thus there is good circumstantial evidence that the chlorophyll and probably the carotenoids are present in the cell in the form of a protein complex. How this structure is related to the lipoids of the plastids is not yet known.

Fluorescence of the pigments in vivo; *sensitized fluorescence*

In the plant the 'position' of the fluorescence band of chlorophyll is further towards the red and at the same time the intensity of fluorescence is reduced to between one-tenth and one-hundredth compared with that in organic solution. In the red and blue-green algae the fluorescence of the phycobilins is also reduced, the band being shifted towards the red.

In the living cell the fluorescence of chlorophyll has been shown to be independent of the wavelength of the exciting light. Thus in *Chlorella* the yield of fluorescence changes very little when the wavelength of the exciting light is changed from a region where it is almost completely absorbed by chlorophyll to a region where carotenoids absorb almost 50% of the incident energy. Thus energy must be transferred from excited carotenoids to chlorophyll. Similarly it has been shown that in diatoms energy can be transferred from fucoxanthol to chlorophyll, and in the red algae from phycoerythrin to chlorophyll. In the latter case it would appear that more than 90% of the energy is transferred and other experiments in which the efficiency of photosynthesis in light of different wavelengths was measured (Chapter 4) confirm this result.

BIOSYNTHESIS OF TETRAPYRROLIC PIGMENTS

In classical work on the biosynthesis of haemoglobin by Shemin, Rittenburg, and others, it was shown how the pyrrol rings of the porphyrin originated from the amino-acid glycine and a four-carbon compound. An immediate precursor of porphyrins is the pyrrol derivative porphobilinogen whose structure was elucidated by Cookson and

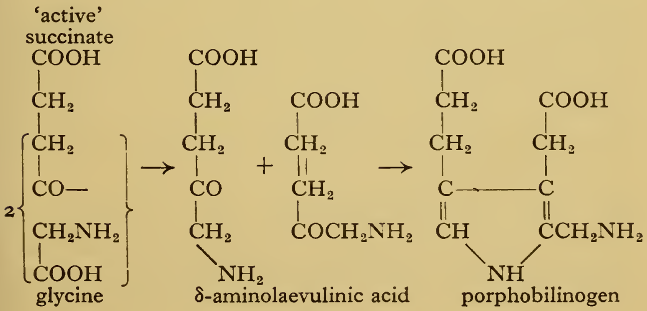


FIG. 3.7

Rimington (1953). Porphyrins are also rapidly formed with cell extracts in presence of δ-aminolaevulinic acid, the substance concluded to be the specific precursor of haem by Shemin and Russell (1953) and by Neuberger and Scott (1953). Porphobilinogen results by the condensation of two molecules of δ-aminolaevulinic acid. The chemical relationships are shown in Fig. 3.7; the four-carbon compound is here represented as an 'active' succinyl group corresponding with the general type of group transfer reactions associated with coenzyme A (p. 101). Porphyrin of the uro-type is very easily formed by the condensation of four molecules of porphobilinogen, in the absence of any biological catalyst. The formation of the porphyrins corresponding to the biological haem compounds depends on decarboxylation and oxidation involving the side chains together with a rearrangement by transfer of a CH_2NH_2 group, prior to the completion of the porphyrin ring structure.

Granick (1950) discovered porphyrin derivatives in mutants from a *Chlorella* strain and has suggested that the biosynthesis of chlorophyll and haem compounds follows a common path until either iron or magnesium enters the tetrapyrrolic pigment nucleus. For example: one mutant was found to accumulate metal-free protoporphyrin, while in another mutant the magnesium derivative of protoporphyrin was found. Oxidation of this last compound could lead to the magnesium-porphyrin corresponding to protochlorophyll; while the final reduction yielding chlorophyll *a* is a process known to occur in the living plant under illumination. This final step in the biosynthesis of chlorophyll *a* has been studied recently in detail by J. H. C. Smith. The reduction of protochlorophyll in the plant can be initiated at relatively low temperatures and depends on the absorption of light by protochlorophyll itself.

The synthesis of chlorophyll in an etiolated leaf is a relatively rapid process and even in a normal mature leaf there is evidence that chlorophyll is being constantly renewed, although the rate of destruction of chlorophyll must be extremely small compared with the rate of photosynthesis.

CHAPTER 4

STUDIES WITH THE LIVING PLANT: THE PHYSIOLOGY OF PHOTOSYNTHESIS

THE early work in photosynthesis briefly described in Chapter 1 was due largely to chemists and contributed to the early development of the chemistry of gases. The next advances in the study of photosynthesis were the result of work in plant physiology. Plant physiology may be defined as the study of the response of living plants or parts of living plants to variable external and internal agencies. The approach is causative; the responses being related in this sense to specified physical or chemical changes in the internal or external environment. The single cell is often taken as the unit of activity but ultimately it becomes necessary to consider the subcellular organization of the cell, especially when we wish to analyse a particular process in chemical terms. Thus the study of fermentation was rapidly advanced by the discovery that fermentation could be made to occur in cell-free extracts. This was the origin both of the name and the concept of an enzyme, i.e. a catalyst which would function apart from the living cell as a whole. From this point in the study of fermentation the biochemical and the physiological aspects seemed to diverge, the latter studying responses of whole living cells whilst the former investigated individual reactions in a cell-free extract obtained in as pure a form as possible. Ultimately however it is clear that the two approaches are complementary and that both are necessary in order to relate the response of the living cell to specified isolated reactions. It must be emphasized that the two approaches differ only in technique and in nothing else. Until very recently it was not possible to obtain any reactions in cell-free extracts which could be brought to bear on the physiology of photosynthesis. Thus up to the middle nineteen-thirties the very large literature of photosynthesis was confined to the study of the effect of different factors on the rate of photosynthesis in the living plant. Consequently there developed only very general ideas as to the chemical mechanism of the process.

The over-all equation given in Chapter I represents the processes of photosynthesis and of respiration and summarizes in a *qualitative* manner the response of the plant to certain external factors, such as carbon dioxide, light, and water supply. In this chapter we shall consider the further advance which was made when these effects were studied quantitatively.

THE FACTORS AFFECTING THE RATE OF PHOTOSYNTHESIS

Interaction of factors

The first object in the study of a process is to find the experimental conditions for the attainment of the maximum rate. Thus the intensity of illumination, the concentration of carbon dioxide, and the temperature may all be varied and all will affect the rate of photosynthesis. F. F. Blackman (1905) demonstrated experimentally that the effect of a variable factor depended on the level at which different constant factors were held, i.e. that there was an *interaction* of factors. This he interpreted as the behaviour of a system consisting of a set of linked reactions any one of which might under appropriate conditions become the main determinant of the over-all rate. Thus for a given light intensity there will be a concentration of carbon dioxide, at which all the available light energy is being utilized. Increase in the concentration of carbon dioxide cannot then appreciably increase the rate of photosynthesis. Similarly, for a given concentration of carbon dioxide there will be a light intensity above which a further increase will not appreciably affect the over-all rate of photosynthesis because the rate is being determined largely by the rate of supply of the carbon dioxide reactant. Under the conditions for maximum rate a small variation in any of the factors will not produce an appreciable effect on the rate. With this understanding of the interaction of factors it will be seen that the study of the effect of varying any individual factor is simplified if all the other factors are kept at some chosen constant level.

The concentration of carbon dioxide. The problem of keeping the concentration of carbon dioxide (or of oxygen) sensibly constant during measurements of photosynthesis was solved by the use of a flow technique first used by Kreusler in 1885. In this method a stream of gas is continually passed over the assimilating material, and the amount of photosynthesis is calculated from the change in carbon dioxide or oxygen concentration of the gas stream. The rate of respiration may be similarly determined when the plant is kept in the dark. Blackman and his collaborators used water plants, but instead of a gas stream circulated a flowing water stream containing dissolved carbon dioxide. The effect of varying the concentration of carbon dioxide was determined, using a light intensity which was so great that further increase did not appreciably increase the observed maximum rate of photosynthesis.

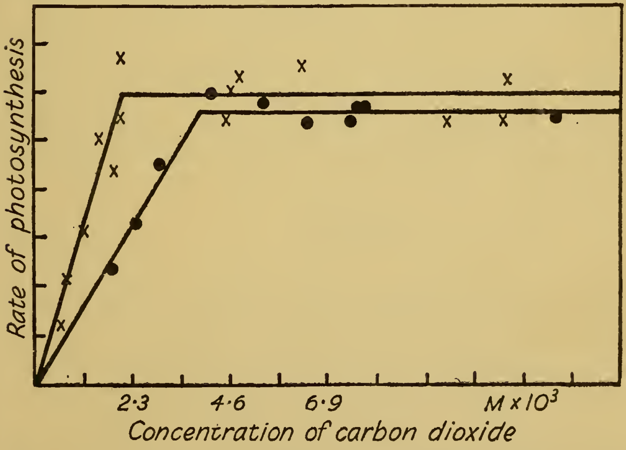


FIG. 4.1. The rate of photosynthesis at high light intensity with different concentrations of carbon dioxide for *Fontinalis antipyretica* (●) and *Elodea canadensis* (×). (Data from Blackman and Smith.) Ord. gm. CO_2/hr./137 cm.^{2} illuminated surface.

Fig. 4.1 illustrates the results obtained by Blackman and Smith (1911) with *Elodea* and *Fontinalis* for the relationship between the rate of photosynthesis at a high light intensity and the concentration of carbon dioxide. The results show that the rate is proportional to the concentration of carbon dioxide over a certain range, but that it is independent of concentration at the highest concentrations. Other workers repeated this type of experiment (sometimes using flowing bicarbonate solutions instead of solutions of carbon dioxide) and found that there was a considerable range during which the rate was neither directly proportional to nor independent of the carbon dioxide concentration. An example of such observations are those of Harder (1921), using the water moss *Fontinalis* (Fig. 4.2). It gradually became clear that a whole variety of types of relationship could be observed and that the observations of Blackman and Smith were appropriate to only one set of experimental conditions.

How such a variety of apparent relationships can come to exist may be considered with reference to enzyme kinetics. Michaelis and Menten suggested that catalysis in an enzymatic reaction was due to the formation of an enzyme-substrate complex which decomposes to give the products of reaction. If S is the concentration of substrate, and the total concentration of enzyme is assumed to be small in comparison to that of substrate, then the relationship between rate and substrate concentration is given by

$$R = \frac{R_{\infty} S}{S + K_C} \tag{4.1}$$

where R_{∞} is the maximum rate and K_C a constant. Let us suppose that in photosynthesis we have a very simple mechanism in which carbon dioxide combines with some enzyme and the rate of breakdown of this complex is determined by the light intensity. Obviously the rate of photosynthesis will be a function both of the concentration of carbon dioxide and of the light intensity. It will only be a simple function of either variable if we limit the experimental conditions. Hence the advantage of studying the effect of light intensity when the concentration of carbon dioxide is so high that practically all the enzyme is combined and of studying the effect of carbon dioxide concentration with an

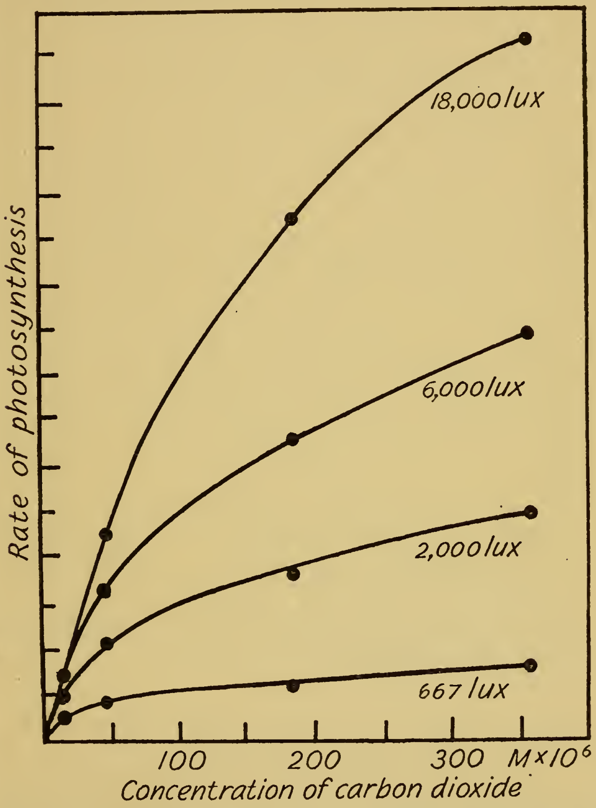


FIG. 4.2. The relationship between rate of photosynthesis and concentration of carbon dioxide at different light intensities in *Fontinalis antipyretica*. (After Harder.)

intensity of illumination so great that further increase does not appreciably affect the rate. Under these conditions we should expect the relationship between rate of photosynthesis and carbon dioxide concentration to approach the type represented by equation (4.1), i.e. a Michaelis relationship. This was first shown by Warburg (1919) to be approximately the case with the unicellular alga *Chlorella* (Fig. 4.3), although there is some departure at the highest concentrations of carbon dioxide. Thus in this plant at least it may be reasonable to suppose that carbon dioxide enters into the photosynthetic process via a single enzymatic reaction.

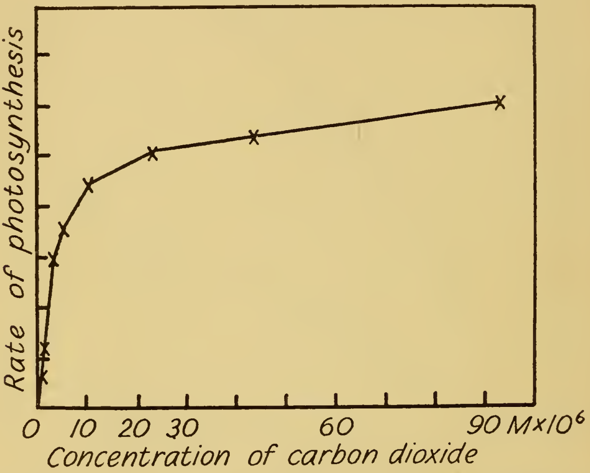


FIG. 4.3. The rate of photosynthesis at high light intensity with different concentration of carbon dioxide in *Chlorella pyrenoidosa*. (After Warburg.)

But we are left with the problem as to the interpretation of the type of relationship observed by Blackman and Smith. The solution to the difficulty becomes apparent when we remember that the experimental curves show the relationship of the rate of photosynthesis to the concentration of carbon dioxide in the solution surrounding the plant, whereas an equation of the Michaelis type refers to the concentration of the carbon dioxide at the place where the reaction is occurring. As we have seen in Chapter 2, the diffusion path between the assimilating cells and the external medium varies considerably in different plants, and thus the concentration of carbon dioxide at the assimilatory centres will vary in different plants even for a fixed external concentration. In *Chlorella* the difference between the 'internal' and the external concentration of carbon dioxide is very small, so that the experimental curve might be expected to approximate to the Michaelis type. In a water plant of more complex structure the diffusion path for carbon dioxide may be much longer, especially in the experiments of Blackman in which some shoots of *Elodea* were placed on top of others. In such a case we must combine with the Michaelis equation, which expresses the rate as a function of the 'internal' concentration, a second expression derived from Fick's law relating the 'internal' to the external concentration. If the length of the diffusion path is L, the coefficient of diffusion K, then if C is the concentration in the external medium and C_s that where the reaction takes place, the rate of diffusion per unit area will be $\frac{K}{L}(C-C_s)$. In addition to this supply of carbon dioxide there will be that from respiration and we shall consider only the simplest case in which all the carbon dioxide from respiration is assumed to be immediately available to photosynthesis. In the steady state the rate of consumption, i.e. the rate of photosynthesis, must equal the total rate of supply of carbon dioxide, so that if the relation between rate of photosynthesis (R) and concentration of carbon dioxide at the reaction centre is of the Michaelis type, then

$$R=\frac{K}{L}(C-C_s)+r=R_\infty\frac{C_s}{C_s+K_c}$$

when r is the rate of production of carbon dioxide in respiration. Hence

$$LR^2-R\,[KC+L.r+KK_c+R_\infty.L]+R_\infty\,[K.C+L.r]=0$$

When the diffusion path becomes very long the term $\frac{K}{L}K_c$

becomes small compared with $(R_{\infty}+r)$ and the equation simplifies to that of two straight lines.

$$(R-R_{\infty})(LR-L.r-KC)=0$$

i.e. $$R=R_{\infty} \quad \textit{or} \quad R-r=\frac{K}{L}.C$$

The relationship between rate and *external* concentration will then be in two parts: (1) a region when the rate of apparent assimilation is proportional to the external concentration and (2) a region where the rate is independent of the external concentration. This type of relationship, which is of the type observed by Blackman *et al.*, represents a limiting case. The slope of the line in the region showing proportionality is $\frac{K}{L}$ so that in this region diffusion is the limiting process and the rate of photosynthesis will be determined by all factors affecting the rate of diffusion. In the region where the rate is independent of concentration the rate will be determined by all factors affecting the rate of consumption of carbon dioxide, e.g. light intensity, temperature, and possibly chlorophyll content. Blackman interpreted his results by ascribing the region of proportionality to one limited by a single *factor*, namely the concentration of carbon dioxide, but analysis clearly shows it to be preferable to refer to the region as limited by a limiting *process*. Thus the data of Blackman on the one hand and those of Warburg on the other represent two extreme cases; one in which the external concentration is very different from the internal and the other in which the two concentrations are nearly the same. The data of other workers are intermediate between these extremes, and represent variations in the length of diffusion path. There may, in addition, be other 'internal' factors which vary from plant to plant and can be related to the form of relationship between rate of photosynthesis and concentration of carbon dioxide (see Briggs and Whittingham, 1952). This possibility will only be appreciated when further work with a greater variety of plants has been done.

In the foregoing discussion the rate of photosynthesis has been considered solely in terms of the concentration of carbon dioxide (together with undissociated carbonic acid). In *Chlorella* the rate of photosynthesis both at high and low concentrations of carbon dioxide appears to be independent of pH. Thus there is no evidence that bicarbonate ions (which are in greater concentration the more alkaline the solution) are decisive in determining the rate of photosynthesis. It seems unlikely that this is true for all plants as there are some indications that in *Scenedesmus*, for example, a more complex situation may exist.

Light intensity. Other measurements have been concerned with the relationship between rate of photosynthesis and the intensity of illumination when the concentration of carbon dioxide is maintained high. Owing to the physical structure of the leaf, the intensity of illumination at a point within the plant will be a function of its depth from the surface; the quality (or colour) of the light will also vary owing to the selective absorption by the cells between it and the light source. Moreover within a single cell the absorption may change as a result of the movement of the chloroplasts, as for example in *Funaria*, in which the chloroplasts assemble on the side of the cell nearest a source of moderate intensity, but in high or more weak intensities distribute themselves in the less brightly illuminated portions (see Fig. 4.4). Precautions must also be taken when using leaves to ensure that the temperature of the leaf does not become higher with higher intensities of illumination. Some of these difficulties are absent or present to a smaller degree when suspensions of algae are used, and we shall consider only measurements with these plants. With *Chlorella* the relationship between rate of photosynthesis and light intensity is again approximately of the Michaelis type, as shown in Fig. 4.5. Just as in the case of carbon dioxide concentration we may then suggest that the simplest possible view is that light energy is involved in a process which results in a chemical change in an acceptor molecule. There is no evidence that chlorophyll undergoes any change during photosynthesis, and hence it is necessary to postulate a substance in addition to chlorophyll which is primarily concerned with the light reaction.

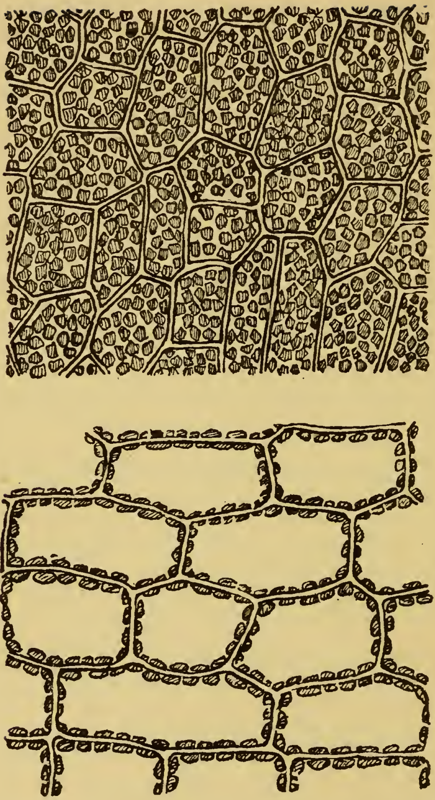


FIG. 4.4. Orientation of chloroplasts of *Funaria* in the light. Upper, with light at right angles to page; lower, in dark. (Drawn from Voerkel.)

We can then write a *minimum* formulation of two processes in photosynthesis as follows:

Process 1. Carbon dioxide $+ A \rightleftharpoons$ carbon dioxide complex
Process 2. Light energy $+ B \rightleftharpoons$ photochemical product
(via chlorophyll)

A and B are unknown substances presumed to exist in the plant cell. The first process is that which determines the relationship between rate of photosynthesis and concentration of carbon dioxide at high light intensities. It is presumed to be a dark process. The second process is that which determines the relationship between rate of photosynthesis and light intensity when the concentration of carbon dioxide is high. This formulation assumes that the rate of these two processes can vary independently so that the rate of the over-all process may be determined by either. The products of photosynthesis are presumed to result from reaction between the carbon dioxide complex and the photochemical product; this is also assumed to be a dark process. A mechanism of

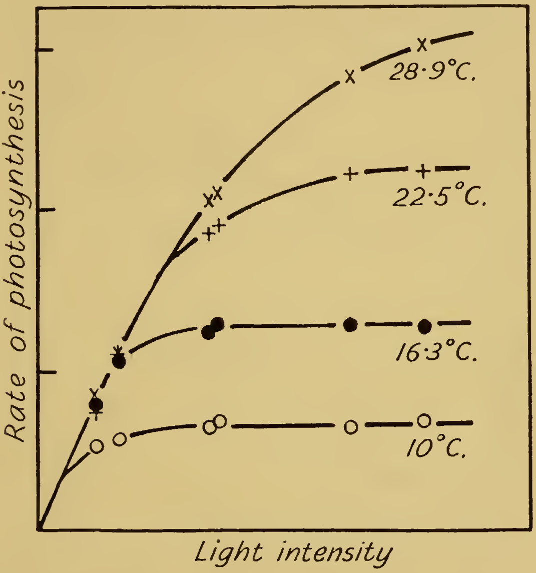


FIG. 4.5. The relationship between rate of photosynthesis and intensity of illumination at different temperatures in *Chlorella.* (After Wassink *et al.*) Highest Light ≡ 1·75 10^4 ergs/cm.^{2}/sec.

this type was suggested by Warburg in 1919 and considered later in more detail by G. E. Briggs (1935).

Temperature. Investigations on the effect of temperature on the rate of photosynthesis provide further evidence for a mechanism of this type. As pointed out by Blackman the maximum rate of photosynthesis at any temperature will only be attained when the concentration of carbon dioxide and the light intensity are both high. Under these conditions Miss Mathaei (1904) found with leaves of cherry laurel and of *Helianthus* a temperature coefficient of the order of 2·1 between 20° and 40° C. Above this temperature the rate of photosynthesis began to decrease due, it was suggested, to a progressive destruction of the photosynthetic mechanism. This decline is thought to commence at temperatures above about 28° C. and to dominate over the accelerating effect of increase in temperature at temperatures above 40° C. The

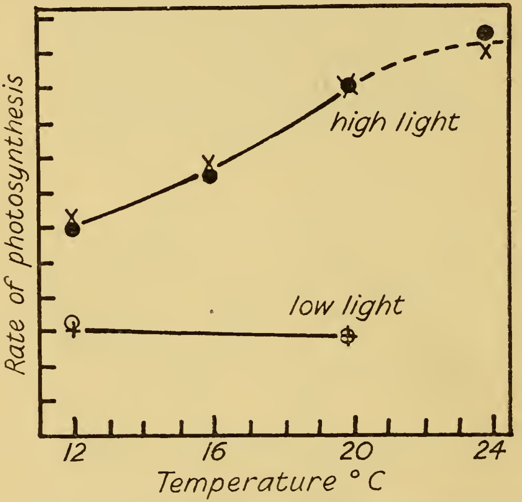


FIG. 4.6. Effect of temperature upon rate of photosynthesis at high (upper) and low (lower) light intensities in *Hormidium*. (After van der Honert.)

temperature coefficient decreases with increasing temperature in the range 0° to 30° C. At low intensities of illumination a smaller temperature coefficient was observed which is consistent with the interpretation that the over-all rate is then limited by the photochemical process, the coefficient of which, by analogy with *in vitro* photochemical reactions, is probably small (see Fig. 4.6). With low concentrations of carbon dioxide, when the rate of the over-all process is determined by some dark process, such as Process 1, the temperature coefficient is greater and values of a fourfold increase for a rise of 10° C. have been reported by Warburg for the alga *Chlorella*. Warburg gave the name of 'Blackman' reaction to the temperature-sensitive dark process responsible for the high temperature coefficient. With a foliage leaf in air the process of diffusion may limit the over-all reaction and in this case of course the apparent temperature coefficient may be appreciably different from that observed with algae.

Poisons. The type of mechanism first proposed by Warburg has also been of value in interpreting the effect of compounds which depress the rate of photosynthesis. Such compounds may be divided into two classes: (1) Poisons which are presumed to combine with some specific component in the photosynthetic mechanism and (2) narcotics which are thought to affect the environment of the reaction, as for example by reducing the 'surface' available for reaction. To determine the class of any particular depressant it is necessary to determine its effect on photosynthesis under a wide range of conditions.

Cyanide, in the form of undissociated hydrocyanic acid molecules, is a powerful depressant of photosynthesis in the class of poisons. In *Chlorella* photosynthesis is almost completely inhibited by concentrations of cyanide too small to affect respiration. In other organisms, e.g. the alga *Scenedesmus*, photosynthesis is inhibited to a smaller extent than respiration and in *Hormidium* it has been claimed (although without subsequent confirmation) that photosynthesis may be even stimulated by dilute concentrations of cyanide. With *Chlorella*, Warburg (1919) found the inhibition of photosynthesis at high concentrations of carbon dioxide was greater at higher light intensities than at low and this led him to suggest that cyanide did not affect the photochemical process in photosynthesis but inhibited the dark reaction (Fig. 4.7). More recently it has been shown that at high intensities of illumination the inhibition of photosynthesis by cyanide is greater at low concentrations of carbon dioxide (Whittingham, 1952) suggesting that the dark process concerned is that in which carbon dioxide is concerned (Process 1). Warburg originally claimed that

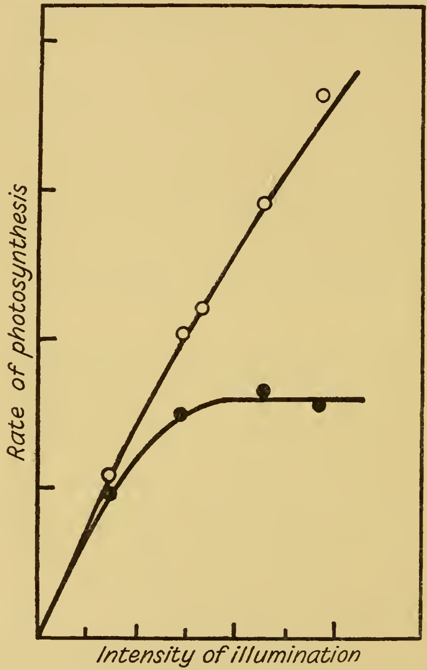


FIG. 4.7. Rate of photosynthesis of *Chlorella* at different light intensities in the presence (●) and absence (○) of cyanide. (After Wassink *et al.*) KCN ≡ 2·3 10^{-5}M.

cyanide would not inhibit photosynthesis below the compensation point and suggested that this was because respiratory intermediates, more reduced than carbon dioxide, could be reduced in the light substituting for the products of process 1. This claim has been shown to be incorrect in at least some strains of *Chlorella*.

Urethanes depress photosynthesis and belong to the class of narcotics. Here again in *Chlorella* photosynthesis is inhibited to a greater extent than respiration but an important difference between its action and that of cyanide is that inhibition of photosynthesis is practically the same for all intensities of illumination. Thus urethane probably interferes in some way with the photochemical process in photosynthesis (although not exclusively, since if it did so the inhibition would be greater at low light intensities) and in agreement with this Warburg claims the inhibition to be independent of the concentration of carbon dioxide.

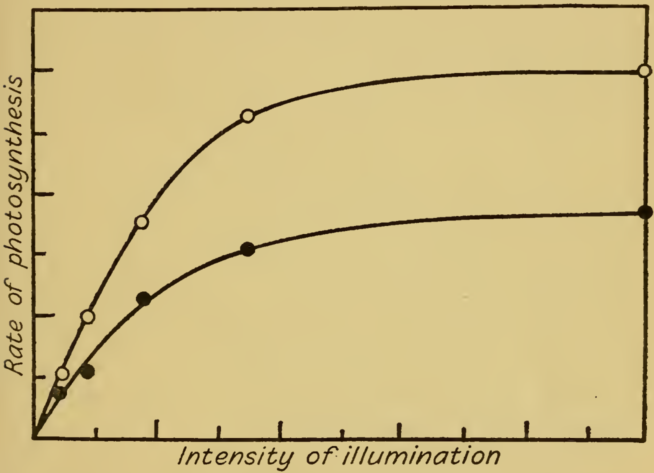


FIG. 4.8. Rate of photosynthesis of *Chlorella* at different light intensities in the presence (●) and absence (○) of hydroxylamine hydrochloride. (After Weller and Franck.)

Since the time of Warburg's experiments the effect of other poisons on photosynthesis has been investigated. Hydroxylamine inhibits photosynthesis in *Chlorella* to about the same extent at all light intensities (Fig. 4.8). It does not inhibit photosynthesis in the photosynthetic bacteria, nor does it inhibit the photochemical reduction of carbon dioxide by green algae using hydrogen (reactions which will be discussed in Chapter 5) to the same extent as photosynthesis. Thus hydroxylamine cannot be classed with urethane as a narcotic but is presumed to be a poison which combines with an enzyme the concentration of which is postulated to be proportional to the light intensity. It is supposed that the enzyme catalyses a dark reaction resulting in the liberation of oxygen. Other poisons include iodoacetate, which like cyanide inhibits photosynthesis to a greater extent at high light intensities, and *o*-phenanthroline which is similar in action to hydroxylamine. As we shall see in Chapter 7 cyanide has no effect on the photochemical reduction reactions catalysed by isolated chloroplasts whereas hydroxylamine and *o*-phenanthroline are powerful inhibitors of these reactions. Since the chloroplast reactions involve only the evolution of oxygen and do not involve carbon dioxide this evidence confirms the view that hydroxylamine and *o*-phenanthroline are effective upon the reaction in which oxygen is liberated.

High concentrations of carbon dioxide (greater than 6%) and very high concentrations of oxygen (greater than 21%) depress the rate of photosynthesis. The depression due to high concentrations of oxygen is only in part due to oxygen consumption in photo-oxidative reactions catalysed by chlorophyll. (*In vitro* photo-oxidative reactions were discussed in Chapter 3.) Such photo-oxidative reactions can be demonstrated *in vivo* with cells in high concentrations of carbon dioxide only at very high light intensities—far in excess of those at which photosynthesis is rapidly approaching 'saturation'. If the concentration of carbon dioxide is reduced photo-oxidative consumption of oxygen occurs at lower light intensities. The depression of photosynthesis due to high oxygen concentrations is greater in magnitude than the actual consumption of oxygen in photo-oxidation and for this reason Franck (1949) has postulated that under conditions of photo-oxidation there is produced in the cell a depressant of photosynthesis. He has assigned a further function to this substance or 'narcotic' which we shall discuss when we refer to work on fluorescence later in this chapter.

Chlorophyll. The effect of the concentration of chlorophyll upon the rate of photosynthesis of the green plant was investigated by Willstätter and Stoll (1918) whose pioneer work on the chemistry of chlorophyll enabled them to estimate accurately the concentration in different plants.

TABLE 4.1

VARIATION IN ASSIMILATION NUMBER IN LEAVES OF DIFFERENT PLANTS

(from Willstätter and Stoll)

Name of plant	Type of leaf	Chlorophyll content mg./10 gm. fresh weight	Assimilation number mg. CO_2/mg. chlorophyll
Ulmus	Normal green	16·2	6·9– 9·9
	Yellow variety	1·2	73 –82
Sambucus nigra	Normal leaf	22·2	6·6
	Yellow variety	0·81	120
Phaseolus	Etiolated	0·7	133
Ampelopsis quinquefolia (autumn leaves)	Young leaf	12·7	7·9
	Old leaf	12·9	0·9

(Measurements in 5% carbon dioxide, high light and at 25° C. See also the data given in the Appendix)

Firstly, they showed that as a result of photosynthesis there was no change in the concentration of chlorophyll in the plant. Then they attempted to determine for a number of different plants, the maximal rate of photosynthesis, in mg. carbon dioxide per hour, per mg. chlorophyll in the tissue —a quantity they called the *assimilation number*. Measurements were made with a flowing gas technique with a high concentration of carbon dioxide and at a high light intensity, although it was not shown in all cases that either of these factors was so high that they were never limiting. With a 'saturating' light intensity the assimilation number measures the activity of the dark processes per unit chlorophyll; if measured with lower light intensities the ratio of the rate of photosynthesis to the concentration of chlorophyll will approach more and more to being simply a measure of the activity of the chlorophyll. Under their conditions Willstätter and Stoll found a considerable range in assimilation number amongst different species of plants. In species with a high assimilation number (or low chlorophyll content) it was found that there was not in general a linear relation between rate of photosynthesis and chlorophyll concentration; for species with high concentrations of chlorophyll the assimilation number tended to be more nearly constant with

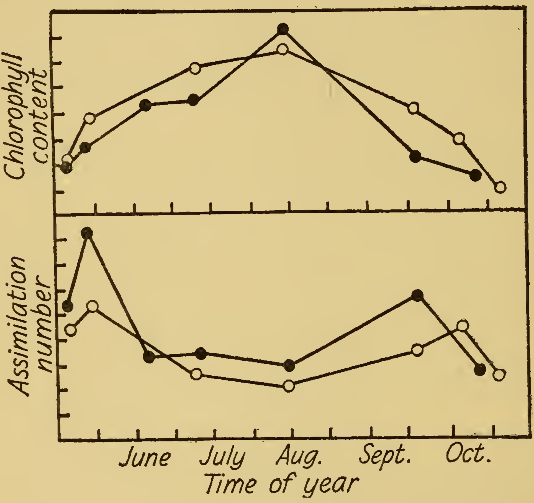


FIG. 4.9. Variation throughout the growing season of chlorophyll content and of assimilation number in *Tilia cordata* (●) and *Acer pseudoplatanus* (○). (After Willstätter and Stoll.)

a tendency to be smaller the greater the chlorophyll concentration. Yellow varieties were found to have higher assimilation numbers than leaves of the corresponding green varieties (Table 4.1). Furthermore, if leaves of a green variety were investigated during the course of a season it was found that as the leaf aged the chlorophyll content increased but the rate of photosynthesis increased to a lesser extent (Fig. 4.9). Thus all these experiments suggested the existence of factors other than chlorophyll which varied from plant to plant and might determine the rate of photosynthesis at high light intensities.

Internal factors other than chlorophyll

The rate of development of such internal factors compared with the rate of development of chlorophyll can be investigated during the greening of etiolated leaves. Willstätter and Stoll found that during greening the assimilation number fell, the first formed chlorophyll resulting in an activity as great as that in some yellow varieties. Earlier work by Irving and later by Briggs (1922) showed that the development of the other internal factors might be more or less rapid than the development of chlorophyll. Thus in *Phaseolus* the young leaves may develop considerable chlorophyll and yet have a low rate of photosynthesis. If however leaves are allowed to develop either in the dark or in the absence of oxygen, so that no chlorophyll is formed, and then allowed to green the photosynthetic activity per chlorophyll is much greater. During the period of growth prior to greening it was supposed that an internal factor essential for photosynthesis had been formed.

Internal factors and mineral nutrition

Chlorophyll deficient plants may be readily produced by culture in media deficient in iron but such plants are likely to be different not only with respect to chlorophyll but also with respect to a whole variety of 'internal' factors. Thus *Chlorella* cultures which are deficient in potassium or manganese show a rapid increase in the rate of photosynthesis, both at high and low light, when the respective elements are supplied. This *immediate* response occurs before any increase in chlorophyll concentration takes place. Similarly during the growth of *Chlorella* an increase in magnesium concentration in a range which does not appreciably increase the chlorophyll content, results in an increase in the rate of photosynthesis. The 'internal' factors important in photosynthesis may be related to the presence or absence of individual enzymes or enzyme systems but there is also the possibility that the rate of photosynthesis may be determined by factors affecting the 'architecture' of the cell. The structure and biochemical organization of the cell will determine the degree of mutual accessibility of the chlorophyll and other components of the photosynthetic system and studies of the quantum efficiency of photosynthesis might be of value in further investigations of chlorophyll deficient plants.

Intermittent illumination. Quantitative study of the internal factors in photosynthesis has been shown to be possible by the use of intermittent illumination. Brown and Escombe and later Warburg measured photosynthesis in intermittent light and found that with high light intensities when cells were rapidly alternated between light and dark the amount of oxygen produced was greater than that produced in a period of continuous illumination of length equal to the light time. More refined experiments were possible in 1932 when Emerson and Arnold (1932) illuminated *Chlorella* with short intense flashes whose duration was about 10^{-5} to 10^{-6} seconds from a neon discharge tube. The dark time between successive flashes was varied and it was found that the yield per flash increased with increase in length of dark time until the dark period was $\frac{4}{10}$th second at 1° C. (Fig. 4.10). Increase in dark time beyond this value did not appreciably increase the yield per flash and this maximum yield corresponded to the evolution of 1 molecule oxygen per 1,000 to 2,000 chlorophyll molecules. This result indicates that there must be some factor other than chlorophyll which limits the amount of energy which can be accumulated by the photosynthetic system during the flash. At first it was suggested that the 2,000 (or so) chlorophyll molecules were arranged in a physical structure, called the 'photosynthetic unit', and that the energy absorbed by all chlorophyll molecules within the unit was contributed to a single molecule of carbon dioxide acceptor. In terms of the mechanism discussed earlier in this chapter a chemical concept of this unit is possible. During the flash, the duration of which is assumed short compared with the half time of all processes other than process 2, all that happens is that a certain amount of photochemical product, equivalent to the amount of substance B, is formed. During the subsequent dark time this product is removed in a slower reaction with the product of process 1. The minimum dark time which must elapse before a second flash can again produce the maximum effect is the time necessary for the virtually complete reaction between these two products. The maximum yield per flash corresponds to the maximum amount of photochemical product formed during the flash and is therefore determined by

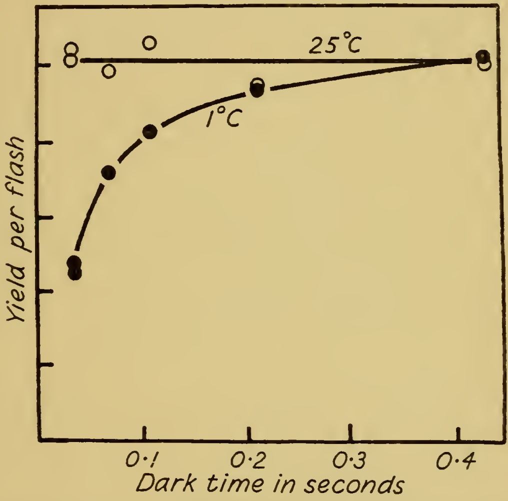


FIG. 4.10. The yield per flash of photosynthesis in *Chlorella* in intermittent light as a function of dark time. (After Emerson and Arnold.) Ord. unit ≡ 10^{-5} μl. O_2/μl. cell/flash.

the amount of B substance. This interpretation, suggested by Briggs (1935) and in a rather different form by Franck and Herzfeld (1941), shows the possibility of measuring the effective amount of an 'internal' factor in terms of some known concentration such as that of chlorophyll. From this reasoning it follows that the hypothetical substance B must be present in an amount of 1 molecule per 2,000 chlorophyll molecules, provided we assume that each molecule of photochemical product produces one molecule of oxygen. If more than one molecule of photochemical product is required the concentration of substance B must be correspondingly greater.

We have already seen that cyanide probably inhibits photosynthesis by slowing down a dark process. Emerson and Arnold investigated its effect on photosynthesis in intermittent light and found that the maximal yield per flash is unchanged but the dark time between flashes necessary to obtain a given fraction of the maximum yield is increased. This is to be expected if cyanide does not alter the amount of B substance present; it might however lengthen the time of reaction of the photochemical product with the product of process 1 by reducing the concentration of carbon dioxide complex.

The experiments of Emerson and Arnold differed from those of Warburg in that the latter used a rotating sector to produce intermittent illumination and consequently the flashes used by Warburg were of longer duration. Briggs pointed out that the yield per flash, expressed on some standard basis such as in terms of the rate of photosynthesis in continuous light, was much greater in the experiments of Warburg than in those of Emerson and Arnold. Experiments using flashes intermediate in duration between those used by Warburg and those used by Emerson and Arnold gave a value for the yield per flash intermediate between the earlier values. With very long flashes and yet longer dark times a maximum yield per flash of one oxygen molecule per chlorophyll molecule was obtained and the dark time was then of the order of 15 seconds. Briggs (1941) therefore postulated the existence of a second dark process possibly involving a second substance present in an amount comparable to the chlorophyll concentration. This might correspond to the hypothetical substance A which combines to form the carbon dioxide complex of the simple mechanism given on p. 50. The longer dark time will then be determined by the velocity constant either of process 1 or of the reaction between the products of processes 1 and 2.

QUANTUM EFFICIENCY

If we assume that the product of photosynthesis is carbohydrate, then in the standard state 115,000 cals. free energy will be required for the reduction of each gram molecule of carbon dioxide. With the concentrations of oxygen and carbon dioxide normally present in air and assuming the concentration of sugar to be 0·05M. the figure becomes 118,000 cals. This energy must be obtained from light and in monochromatic red light, say 6,780Å, the maximum of chlorophyll absorption, it will be available in units or mole quanta each of 41,000 cals. Thus for each gram molecule of carbon dioxide reduced at least three mole quanta will be required. As we have seen, the maximum efficiency will only be observed when the concentration of carbon dioxide is so high that it does not limit the rate and when the light intensity is small, i.e. in that region of the light curve where the slope is a maximum. With leaves of higher plants it is not easy to determine the light energy absorbed by the plant owing to the difficulty of determining the total reflected and transmitted light. Briggs (1929) and Gabrielson (1935) both obtained values of about 12 quanta red light/molecule oxygen. With suspensions of algae more precise measurements are possible by the use of an integrating sphere. This consists of a totally reflecting surface completely surrounding the algal suspension, except for a very small hole through which the incident beam enters and a second hole in which the detecting instrument is placed. The scattered light is reflected round the sphere until an average sample falls on the detector. A zero reading on the detector is obtained by replacing the algal suspension by a totally absorbing system such as a suspension of indian ink. With suspensions which absorb only part of the incident light a variety of methods of measuring the rate of photosynthesis have been used including manometry, gas analysis, and calorimetric methods. All the results are in good agreement and indicate a value not very different from that obtained for leaves, namely, about 8 to 10 quanta/molecule oxygen. Other observers have avoided the problem of scattered light by using suspensions of algae so dense as to absorb nearly all the incident light. With such suspensions the only measurements have been manometric and have been largely restricted to short alternate periods of light and dark when we are least certain as to the nature of the products of the reaction. Indeed it is clear that the first measurements of Warburg and Negelein (1920), which claimed an observed efficiency in *Chlorella pyrenoidosa* of only 4 quanta/molecule

TABLE 4.2

EFFICIENCY OF PHOTOSYNTHESIS IN *Chlorella pyrenoidosa* IN RED LIGHT

Name of investigator	Method of measurement	Quantum efficiency mol. O_2/quantum	Quantum requirement quanta/mol. O_2	Light intensity used ergs/cm.^{2} sec.
Moore and Duggar (1949)	Polarograph	0·074 0·12	13·5 8·3	1,958 783
Arnold (1949)	Calorimetric	Maximum value 0·108	9·2	90 (approx.)
Manning (1938), Stauffer, Duggar, and Daniels	Gas analysis	Maximum value 0·1	10·0	1,000–1,750
Emerson and Lewis (1939)	Manometry Dense suspension	0·063 0·113	16·0 9·0	8,000 1,400
Rieke (1939)	Manometry Thin suspension	0·084 0·079	11·9 12·7	1,900 4,400
Warburg (1948)	Manometry	0·25	4·0	—

oxygen, were in error as shown by Emerson and Lewis due to the incorrect assumption that the assimilatory quotient was unity during such short periods as 10 minutes. More recent experiments by Warburg, which have avoided many of the sources of criticism levelled at his earlier work, confirm his previous finding of 4 quanta/molecule oxygen. Later Burk and Warburg (1951) claim to have observed a partial process in photosynthesis requiring only one quantum/molecule of oxygen. This was observed when cells were subjected to alternate periods of light and darkness of 1½ minutes duration. The reliability of these measurements is still uncertain and their interpretation will be discussed in the last chapter. It appears most likely that in a steady state 8 to 10 quanta of red light are absorbed per molecule of oxygen produced. If each molecule of oxygen corresponds to the reduction of a molecule of carbon dioxide to carbohydrate the thermodynamic efficiency must therefore be of the order of 30%. Higher values are not excluded but have been found in the past by only a few investigators.

For plants growing in the field the efficiency is much smaller, being of the order of some 3% of the absorbed light or about 2% of the incident light energy. Brown and Escombe (1905) estimated that for *Helianthus annuus* 1·0% of the total absorbed solar radiation (about half of which is in the visible) was used for photosynthesis and about 70% or more is concerned in the vaporization of water.

Photosynthetic efficiency in light of different wavelengths. The effect of the colour of light upon photosynthesis was investigated during the second half of the nineteenth century and the results of Englemann (1882 onwards), whilst hotly disputed by his contemporaries, are now being vindicated by present-day researches. Englemann was the first to show the general similarity between the amount of photosynthetic activity in a spectral region and the absorption of light in that region by the plant pigments. The *action spectrum* relates the amount of photosynthetic activity for an equal number of incident quanta to the wavelength of the incident light. Measurements should be made with intensities of illumination so low that there is an increase in the rate of photosynthesis proportional to each increase in light intensity for all wavelengths, i.e. at no wavelength is the light 'saturating'. Then provided the absorbed energy is equally available to the photosynthetic system the action spectrum should parallel the absorption spectrum. In as far as this is found to be untrue we can deduce from studies comparing the action spectrum of photosynthesis with the absorption spectrum of the plant the relative photosynthetic efficiency of light absorbed by different pigments.

As we said in Chapter 3 it is not easy to deduce from the absorption of the living cell the relative absorption by each of the constituent pigments even when the absorption curves of the latter are known. Further conclusions can be made only concerning the relative activity of the different pigments. For this reason it is customary to assume that the photosynthetic efficiency of chlorophyll is independent of wavelength, i.e. of quantum size. Throughout most of the visible spectrum this hypothesis appears to be correct but in the far red, beyond 680 mμ, there is a rapid decline in photosynthetic activity even within the region where the absorption by chlorophyll is still appreciable. A satisfactory explanation of this observation has not yet been suggested.

The variation of quantum efficiency with wavelength in *Chlorella* was investigated by Emerson and Lewis (1943) whose data are shown in Fig. 4.11. There is a marked drop in the photosynthetic yield in that region where the carotenoids absorb. The decrease is not so great as is to be expected from the relative absorption by the pigments, for where the carotenoids absorb about 50% of the incident energy the decline in photosynthetic yield is only about 25%. Thus it may be concluded that light absorbed by carotenoids is about 50% as efficient for photosynthesis as that absorbed by chlorophyll. Whether this decreased efficiency is due to some of the carotenoids in *Chlorella* being completely inactive or to a generally lower efficiency of all the carotenoids is not yet known. In the diatoms it has been shown that light absorbed by fucoxanthol is almost equally as effective as that absorbed by chlorophyll (Dutton and Manning, 1941; Tanada, 1951). Of the phycobilins, Emerson and Lewis (1942) have shown in *Chroococcus* that light absorbed by phycocyanin is almost as effective as that absorbed by chlorophyll. In the red algae Haxo and Blinks (1950) have shown that light absorbed by phycoerythrin appears to have an even greater efficiency than light absorbed by chlorophyll. This however is probably due to the fact that in the species studied light absorbed by chlorophyll itself was used with poor efficiency, thus raising the question, not yet fully answered, as to whether all of the chlorophyll in a cell is equally efficient photosynthetically.

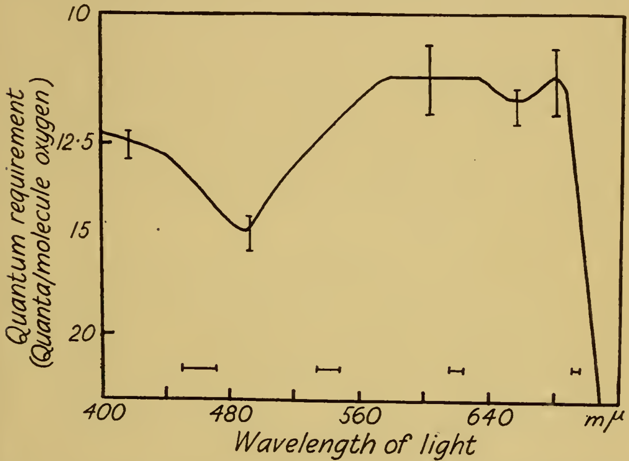


FIG. 4.11. Quantum efficiency of *Chlorella pyrenoidosa* as a function of the wavelength of light. (After Emerson and Lewis.) Variation in data shown by vertical lines; band width in different spectral regions by horizontal lines.

Hence there is good evidence that the light absorbed by pigments other than chlorophyll can be utilized for photosynthesis. Since chlorophyll *a* is the only pigment common to all photosynthetic organisms it has been suggested that it is the only pigment which directly donates energy to the photosynthetic reactants, the other pigments first transferring their excitation energy to it. This may be correlated with the fact that its absorption band is the farthest to the red of the chief pigment components. In the red algae in which chlorophyll *d* is present, the absorption band of which is farther into the red, there is some evidence that when transfer of energy occurs from chlorophyll *a* to chlorophyll *d* there is a loss of energy from the photosynthetic system. On the other hand, when energy is transferred from phycoerythrin to chlorophyll *a* it is suggested that transfer occurs to a portion of the chlorophyll *a* which does not 'leak' appreciably to chlorophyll *d*. This hypothesis, based on the observation that phycoerythrin-sensitized fluorescence of chlorophyll is stronger than its directly excited fluorescence, also offers an explanation of the relatively poor efficiency of light absorbed by chlorophyll as compared with the greater efficiency of light absorbed by the phycobilins in some red algae (Duysens, 1952).

STUDIES OF THE INTENSITY OF FLUORESCENCE

In addition to studies of the sensitization of fluorescence of one pigment resulting from absorption of light by a different pigment, described in Chapter 3, attempts have also been made to correlate changes in the intensity of fluorescence with changes in the rate of photosynthesis. In the preceding section we have seen that perhaps as much as 30% of the absorbed light energy finally is converted into chemical energy. The remaining 70% is dissipated largely as heat but a small fraction is re-emitted as fluorescent light. In the living cell the yield of fluorescence is of the order of 1·0 to 0·1% of the incident light energy. Kautsky discovered changes in the intensity of fluorescence of chlorophyll during the induction phase of photosynthesis and showed that there was in general an inverse relationship between the rate of photosynthesis and the intensity of fluorescence. Thus, after a short aerobic dark period, whilst the rate of photosynthesis increased during the first minutes of illumination the intensity of fluorescence fell from an initial high value to about half of this. Similar results were found by McAlister and Myers (1940). A simple interpretation was suggested, namely that there was a direct competition between fluorescence and photochemical reaction. Another example of a change in rate of photosynthesis being accompanied by an opposite change in fluorescence intensity can be observed during a steady state of photosynthesis. If the temperature of a leaf is lowered, after an initial transitory 'burst' of fluorescence, the fluorescence intensity remains higher corresponding to the lowered rate of photosynthesis. The existence of such a 'burst' phenomenon together with a number of other observations led Franck (1949) to doubt the correctness of this simple view of 'anti-parallelism'. Franck, French, and Puck (1941) investigated the variation in fluorescence yield as a function of light intensity. At low light intensities the yield was at first constant but as the intensity was increased the fluorescence yield increased until it finally attained a new steady value some 70% greater. The intensity at which the higher yield is attained may be higher than that at which photosynthesis approaches its 'saturation' rate, as in *Chlorella*, or it may be about the same intensity, as in *Hydrangea*. The transition from the lower to the higher yield occurs at lower light intensities the lower the concentration of carbon dioxide or in the presence of cyanide. Similarly, as shown by Wassink *et al.*, in the purple photosynthetic bacteria the transition occurs at lower intensities the lower the concentration of hydrogen donor. An exception was found however in the diatom *Nitzschia* in which Wassink and Kersten (1946) observed a decrease in fluorescence yield with increasing light intensity. It thus became apparent, as Franck has frequently pointed out, that factors other than a simple competition between fluorescence and photosynthesis must be considered. Fluorescence can only compete with the primary photochemical process in photosynthesis. Some factors, however, e.g. cyanide, affect the over-all process by changing the rate of a dark process and thus must affect the concentration of intermediates associated with the chlorophyll. This might easily result in a change in the rates of reactions resulting in dissipation of energy, e.g. as heat. Since these involve a large proportion of the energy available, a small percentage change in their activity will result in a large percentage change in fluorescence. Thus if the rates of the processes resulting in dissipation increase, when the rate of photosynthesis decreases, the fluorescence may also decrease. In addition to the effect of intermediates of photosynthesis upon the processes of dissipation, Franck has postulated the formation by the cell metabolism of other substances (called by him 'narcotics') which are supposed to inhibit both energy dissipation and photo-chemical reactions. These substances are considered to be produced by the cell in the dark, especially under anaerobic conditions, and during photo-oxidation. They are thus of particular importance during the induction phase and under conditions of low concentrations of carbon dioxide or very high light intensities. Further studies of fluorescence in the plant may be expected to reveal complexities of which there is yet only a vague understanding. Much work remains before a unifying interpretation of all the data can be hoped for.

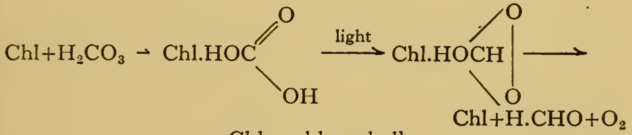
PRODUCTION OF LIGHT BY GREEN PLANTS

Using both algae (*Chlorella*, *Scenedesmus*, and *Stichococcus*) and higher plants, Strehler and Arnold (1951) showed that after illumination had ceased, light was given out by plants for a period which may be as long as two minutes, the half time being of the order of 1 to 2 seconds. The intensity is of the order of 10^{-6}th that of the exciting light and its discovery was made possible by the use of sensitive photomultiplier tubes. Heat irreversibly destroys the ability to luminesce; luminescence is inhibited by compounds which inhibit photosynthesis, e.g. cyanide, azide, and hydroxylamine. Luminescence is 'saturated' by exciting intensities of the same order as those required to saturate photosynthesis. High concentrations of carbon dioxide (7·5%) depress the emission of light but the depression is reversible. Chloroplast suspensions show similar effects to whole cells (Strehler, 1951). Thus the properties of luminescence are different from those to be expected if the light emission were phosphorescent; it is thought probable that light originates from the reversal of some steps of the photosynthetic mechanism.

KINETIC FORMULATIONS OF PHOTOSYNTHESIS BASED ON RESPONSES OF THE PLANT

The slow development of activity in the study of the biochemistry of photosynthesis meant that precise ideas as to the chemical mechanism of the process involved could not at first be formulated. From the observed physiological responses of the plant, physiologists suggested a possible mechanism of the process in terms of the minimum number of arbitrary postulates.

The simplest type of mechanism postulated that photosynthesis was a direct 'photolysis' of carbon dioxide catalysed by chlorophyll. But as we have seen in this chapter a one-step process is too simple to provide an interpretation of the effect of external factors on the rate of the overall process. Willstätter and Stoll formulated a mechanism to take account of some of these effects. They suggested a three-step process involving (1) a reaction between carbon dioxide and chlorophyll to form a chemical complex, (2) the absorption of light by this complex resulting in the formation of a peroxide, and (3) a dark reaction in which oxygen is evolved from the peroxide with the liberation of free formaldehyde. The third reaction was regarded as an enzymic process involving an 'internal factor' of a type which had been postulated with reference to work on the assimilation number, and which has been discussed earlier in this chapter.



$$\mathrm{Chl}+\mathrm{H_2CO_3} \rightarrow \mathrm{Chl.HOC}\begin{matrix}\diagup \mathrm{O} \\ \diagdown \mathrm{OH}\end{matrix} \xrightarrow{\text{light}} \mathrm{Chl.HOCH}\begin{matrix}\diagup \mathrm{O} \\ \diagdown \mathrm{O}\end{matrix} \longrightarrow \mathrm{Chl}+\mathrm{H.CHO}+\mathrm{O_2}$$

Chl = chlorophyll

A slightly different mechanism was first proposed by Warburg (1919), later abandoned by him in 1924, but recently more fully developed by Briggs, in 1935 and 1941. In Briggs' formulation the chemical nature of the reactants is not specified. Essentially there are three reactions, (1) the formation of a complex between carbon dioxide and some intracellular substance not necessarily chlorophyll, (2) the activation of this complex not directly by light but by a product of the light reaction, the amount of this product limiting the rate of supply of energy to the photosynthetic system at high light intensities, and (3) the breakdown of the activated complex with the formation of reduced carbon compounds. This mechanism differs from that of Willstätter and Stoll in the introduction of an intermediate between the light process and the carbon dioxide intermediate. An estimate of the amount of this intermediate B (in terms of chlorophyll) can be obtained from the results of experiments with flashing light and its concentration is found to be of the order of 1/2,000th that of chlorophyll. The catalyst A has been shown to be present in a concentration approximately equal to that of the chlorophyll. So far these substances are identified solely in terms of their kinetic properties, their chemical nature being completely unspecified.

$$\left.\begin{array}{r} \mathrm{CO_2+A} \rightleftharpoons \mathrm{A}c \\ \mathrm{Light+B} \rightleftharpoons \mathrm{B'} \end{array}\right\} \rightarrow \text{photosynthetic product}$$

(via chlorophyll)

Franck and Herzfeld (1941) put forward an essentially similar theory but in which it was supposed that carbon dioxide or a derivative of carbon dioxide formed a chemical complex with chlorophyll. Three types of process were proposed similar to those of the earlier formulations. The first process involved the formation of a carbon dioxide complex with a substance each molecule of which was presumed to be associated with at least one molecule of chlorophyll. The photochemical process was suggested as a two-step process, the first quantum resulting in the transfer of a hydrogen atom from chlorophyll to the carboxyl group of the complex, the second resulting in the regaining of the hydrogen atom by chlorophyll from a donor which was ultimately water. Since four hydrogen atoms are transferred per molecule of carbon dioxide reduced to carbohydrate such a mechanism as this requires eight photochemical steps, i.e. eight quanta as a minimum for the reduction of one molecule of carbon dioxide. All photochemical intermediates are postulated as back reacting unless 'stabilized' by a catalyst, catalyst B, whose amount limits the rate of photosynthesis at high light intensities and therefore determines the maximal yield in brief light flashes. After effecting 'stabilization' this catalyst is postulated as becoming inactive and only becomes active again as a result of a spontaneous unimolecular reaction. The velocity constant of the latter determines the dark time necessary between flashes for the attainment of maximum yield.

1. $$\mathrm{RH}+\mathrm{CO_2} \xrightarrow{\text{catalyst A}} \mathrm{R.COOH}$$

2. $$\mathrm{H.Chl.R.COOH}_n \underset{\text{catalyst B}}{\overset{\text{light}}{\rightleftharpoons}} \mathrm{Chl.R.COOH}_{n+1}$$

$$\mathrm{R'OH}+\mathrm{Chl.R.COOH}_{n+1} \xrightarrow[\text{catalyst B}]{\text{light}} \mathrm{H.Chl.R.COOH}_{n+1}+\mathrm{R'O}$$

$$n = 1, 2, 3,$$

3. $$4\mathrm{R'O}+2\mathrm{H_2O} \rightarrow 2\mathrm{R'OH}+2\mathrm{R'OOH}$$
$$2\mathrm{R'OOH} \rightarrow 2\mathrm{R'OH}+\mathrm{O_2}$$

These three theories all agree in the division of the photosynthetic process into three distinct steps. All postulate a role for other substances in addition to chlorophyll each of which may limit the rate under certain conditions.

The further development of the subject and the more specific interpretation of these general ideas as to the mechanism of photosynthesis came in the first place from studies in the comparative biochemistry of photosynthesis and later from studies with radioactive tracers and with chloroplasts isolated from the cell.

CHAPTER 5

THE COMPARATIVE BIOCHEMISTRY OF PHOTOSYNTHESIS AND OF CARBON DIOXIDE FIXATION

UNTIL the end of the nineteenth century there appeared to be a sharp distinction between the green plants, on the one hand, which were able to develop in the light on a completely mineral medium—the autotrophs—and all other plants together with animals which required a supply of organic substances for growth—the heterotrophs. This simple distinction has become less useful as a result of the discovery of the wide range of biochemical activity exhibited by bacteria.

In 1887 Winogradsky discovered a group of organisms which could assimilate carbon dioxide in the dark. This they could do only in the presence of hydrogen sulphide and as a result of the reaction refractile globules of sulphur were deposited within the cells; in the absence of sulphide the sulphur globules disappeared, sulphate was liberated into the culture medium, and eventually the cells died. A few years later Winogradsky discovered a different group of organisms which grew only in the presence of carbon dioxide and ammonia or nitrite. Both groups of organisms were colourless and, although they grew in the dark, were autotrophic. The energy necessary for the reduction of carbon dioxide was obtained from the simultaneous oxidation of a simple inorganic chemical compound, such as sulphide or sulphur or ammonia or nitrite, a type of metabolism described as chemosynthetic or chemo-autotrophic.

Prior to Winogradsky's discovery of colourless bacteria utilizing sulphur, Englemann in 1883 had described pigmented bacteria which assimilated carbon dioxide in the presence of sulphide but which apparently needed light. In subsequent work several difficulties arose with regard to these bacteria since it was difficult to understand why, if the bacteria were photosynthetic, they should also require a supply of sulphide; furthermore, in the light no oxygen production could be detected and indeed the cells grew only under strictly anaerobic conditions. Since they required sulphide there was naturally a tendency to suppose they were related to the sulphur-utilizing organisms of Winogradsky but differed in the presence of a pigment imparting a purple coloration and in the necessity of light for growth. Later yet other purple bacteria were found by Molisch which required both light energy and a supply of organic substances for growth. Molisch suggested that in these organisms light was necessary for the assimilation of the organic substances.

The difficulties which beset investigators in this field were in part due to the fact that the organisms were not obtained in pure culture. Later when this was achieved, van Niel (1941) was able to make clear the inter-relationship between the purple bacteria and the sulphur bacteria. He was able to distinguish these bacteria as members of three groups, namely,

1. The chemosynthetic colourless bacteria of Winogradsky which assimilate carbon dioxide as a result of the oxidation of sulphide.
2. Photosynthetic bacteria which do not require organic growth factors and utilize inorganic compounds in the light (Thiorhodaceae). These fall into two groups:—
    - *a.* green bacteria which assimilate carbon dioxide in light in presence of sulphide and cannot utilize organic compounds;
    - *b.* purple bacteria which utilize sulphide in the light but can in addition utilize some organic compounds. (Englemann's organisms.)
3. The photosynthetic purple bacteria of Molisch which require organic growth factors and in general use organic hydrogen donors for assimilation of CO_2 in light (Athiorhodaceae).

It is found that H_2 can act as H donor for many of the organisms in groups 2*b* and 3. Between the main groups of Thiorhodaceae and Athiorhodaceae, *Rhodomicrobium vannielii* occupies an intermediate position since it requires no growth factors but utilizes organic compounds in the assimilation of carbon dioxide in the light.

THE PHOTOSYNTHETIC PURPLE SULPHUR BACTERIA (THIORHODACEAE)

The Thiorhodaceae will grow on strictly inorganic media in which carbon dioxide is the sole carbon source. Growth will only occur under anaerobic conditions and in an amount proportional to the quantity of sulphide available (Table 5.1). Even using luminescent bacteria, the most sensitive way of detecting minute amounts of oxygen, no oxygen production in the light has ever been observed. The sulphide is oxidized to sulphur, which is deposited within the cells, and may be ultimately converted to sulphate. Wieland and later Kluyver considered that all the reactions concerned in living organisms were oxidation-reduction processes involving a transfer of hydrogen or of electrons, as for example:—

TABLE 5.1

THE SULPHIDE REQUIREMENT OF THIORHODACEAE

(*from van Niel* (1941))

Number of days' growth	Hydrogen sulphide consumed mg.	Carbon dioxide consumed mg.
27	8·8	20·7
34	17·2	39·2
42	18·7	46·8
48	18·7	46·9
14	12·7	32·1
24	24·9	63·8
25	24·8	63·1
47	24·8	63·9

$$AH + B \rightarrow A + BH$$

van Niel's experiments showed that the photosynthetic reaction of the purple bacteria could be represented by the equation:—

$$2H_2S+CO_2 \rightarrow \text{photosynthetic product } (CH_2O)+2S+H_2O$$

He therefore suggested that in these organisms the hydrogen sulphide simply acted as a hydrogen donor, itself becoming oxidized to free sulphur. This might be compared to the photosynthetic reaction in green plants which can be represented as:—

$$2H_2O+CO_2 \rightarrow \text{photosynthetic product } (CH_2O)+O_2+H_2O$$

where it may be suggested by analogy that water serves simply as a hydrogen donor. Thus photosynthesis may be considered as an oxidation-reduction reaction. This hypothesis offers an explanation both for the requirement of sulphide for carbon dioxide reduction and for the fact that the product of the reaction in the bacteria is not oxygen but sulphur. It is now known that a variety of hydrogen donors can be used by the Thiorhodaceae, including thiosulphate, selenium compounds, and in some purple bacteria simple organic substances.

$$5H_2O+2CO_2+Na_2S_2O_3 \rightarrow 2(CH_2O)+2H_2O+2NaHSO_4$$
$$3CO_2+8H_2O+2Se \rightarrow 3(CH_2O)+3H_2O+2H_2SeO_4$$

The green sulphur bacteria (Chlorobacteriaceae) are similar to the purple sulphur bacteria, differing in the lack of the pigments imparting purple coloration. In this group the sulphur granules are generally deposited outside the cells.

THE PURPLE NON-SULPHUR BACTERIA (ATHIORHODACEAE)

The Athiorhodaceae (with the exception of some strains of *Rhodopseudomonas palustris*) are unable to utilize reduced sulphur compounds but grow in the presence of simple organic substances such as organic acids. All require the addition of growth factors; some require biotin or thiamin or *p*-aminobenzoic acid, or combinations of thiamin and biotin, or thiamin, biotin, and nicotinic acid. This requirement distinguishes the Athiorhodaceae from the Thiorhodaceae.

The organisms were at first regarded as heterotrophic, directly assimilating organic compounds in the light, but van Niel suggested that there was another possibility, namely that the organic substances might act simply as hydrogen donors in a photosynthetic process. This was shown by Foster to be the case for a substrate such as isopropanol which was quantitatively converted to acetone at the same time that an equivalent quantity of carbon dioxide was consumed, in accordance with the equation:—

$$2CH_3CHOHCH_3+CO_2 \rightarrow (CH_2O)+2CH_3COCH_3+H_2O$$

Some results are given in Table 5.2. The work was subsequently confirmed by Seigel and Kamen using labelled carbon dioxide and labelled alcohol. On the other hand work with labelled acetate showed that acetate does not serve exclusively as hydrogen donor but is in part directly assimilated by the cell without passing through the stage of CO_2. Thus certain substrates are used entirely as hydrogen donors whereas other substrates are in part used as hydrogen donors to carbon dioxide and in part assimilated heterotrophically. Indeed some strains of Athiorhodaceae can live heterotrophically in the dark under aerobic conditions by oxidizing organic substance whereas in the light under anaerobic conditions they utilize a photosynthetic mechanism; i.e. these strains are facultatively anaerobic.

TABLE 5.2

UTILIZATION OF ISOPROPANOL BY A STRAIN OF PURPLE NON-SULPHUR BACTERIA

(*from van Niel*)

Moles isopropanol consumed	Moles acetone produced	Moles carbon dioxide consumed	Molar ratio isopropanol/CO_2
34·19	35·00	15·45	2·22
48·85	51·70	24·10	2·02
77·00	78·45	38·40	2·00
82·90	82·50	39·10	2·12
90·20	91·65	45·70	1·97
110·4	113·0	56·00	1·97

HYDROGEN EVOLUTION AND NITROGEN FIXATION IN ATHIORHODACEAE

Under anaerobic conditions growth in the light of Athiorhodaceae which utilize organic compounds more oxidized than carbohydrate results in the production of carbon dioxide; if organic compounds more reduced than carbohydrate are utilized carbon dioxide is consumed and must be supplied to obtain good growth. This may be represented in a general equation for either monobasic or dibasic acids:—

monobasic acid

$$C_nH_{2n+1}COOH+\frac{n-1}{2}CO_2+\frac{n-1}{2}H_2O \rightarrow \frac{3n+1}{2}[CH_2O]$$

dibasic acid

$$C_nH_{2n}(COOH)_2+\frac{n-3}{2}CO_2+\frac{n-1}{2}H_2O \rightarrow \frac{3n+1}{2}[CH_2O]$$

Thus for the monobasic acid, formic acid ($n = 0$), carbon dioxide is produced whereas for acetic ($n = 1$) and fatty acids $n \gtreqless 2$ carbon dioxide will be consumed. However, in *Rhodospirillum rubrum* (Athiorhodaceae), in addition to the production of carbon dioxide, hydrogen is also produced in the light under anaerobic conditions in the presence of a variety of organic substances such as fumarate, malate, pyruvate, acetate, or oxalacetate. This invalidates the simple equation given above. Further it has also been shown that *R. rubrum* fixes molecular nitrogen when illuminated in a medium containing malate, biotin, and mineral salts, in which case the photochemical production of hydrogen is decreased as is also to a less extent the evolution of carbon dioxide. Addition of ammonia also decreases the production of hydrogen and carbon dioxide although in this case the decrease is greater.

THE GENERALIZED NATURE OF THE LIGHT REACTION IN PHOTOSYNTHESIS

According to van Niel a generalized equation for photosynthesis can be written as $CO_2+2H_2A \rightarrow (CH_2O)+H_2O+2A$, thus representing photosynthesis as an oxidation-reduction reaction in which carbon dioxide is reduced by an oxidizable hydrogen donor. According to this equation referred to the green plant, i.e. $CO_2+2H_2O \rightarrow CH_2O+H_2O+O_2$, water might be considered to act only as a hydrogen donor. Consequently it would be expected that the oxygen liberated should come entirely from the water molecules. By the use of the isotope ^{18}O which can be introduced into either the water or the carbon dioxide molecules (as bicarbonate or carbonate) the composition of the oxygen evolved may be compared with that of the reactants. The results are consistent with the interpretation that the whole of the oxygen originates from the water molecules (Table 5.3) and that the equation for green plant photosynthesis should be written:—

$$CO_2+2H_2O^* \rightarrow (CH_2O)+H_2O+O_2^*$$

TABLE 5.3

THE COMPOSITION OF THE OXYGEN EVOLVED IN PHOTOSYNTHESIS IN *Chlorella*

(*Ruben, Randall, Kamen, and Hyde* (1941))

Per cent ^{18}O in bicarbonate and carbonate	Per cent ^{18}O in water	Per cent ^{18}O in oxygen evolved
0·61	0·85	0·86
0·50	0·20	0·20
0·40	0·20	0·20

A complication arises in the interpretation of these experiments since it is possible that exchange of oxygen isotope may occur between carbon dioxide and water molecules and that the oxygen liberated during photosynthesis may equilibrate with the water present in the system either outside or within the cell. *In vitro* these reactions are slow compared with the rate of photosynthesis but the possibility arises that the exchange reactions may be catalysed by living cells. Subsequent attempts to repeat this experiment modifying the procedure in such a way as to minimize the importance of exchange reactions have not produced indisputable evidence either for or against the theory that all the oxygen arises from water. Further evidence which makes this interpretation of green plant photosynthesis still more probable has come from work with isolated chloroplasts which will be discussed in Chapter 7.

At first sight it might appear that the energy required for the reduction of carbon dioxide by different organisms would differ according to the hydrogen donor utilized. Thus the reduction of carbon dioxide by hydrogen sulphide with the formation of carbohydrate and sulphur requires 17,400 cals. per mole, by hydrogen gas only some 2,700 cals., whereas with water 112,000 cals. are necessary. In spite of these differences in the free energy of reaction it has been found experimentally that the number of light quanta absorbed per mole of carbon dioxide reduced is approximately the same in the purple sulphur bacteria (Wassink, Katz, and Dorrestein, 1941), the green sulphur bacteria (Larsen, Yocum, and van Niel, 1952), and usually in the green plant (see Chapter 4), namely about 10 quanta. This led van Niel to suggest that in all of these processes the primary photochemical reaction is the same and consists in the removal of hydrogen from water with the formation of a hydrogen donor which ultimately donates its hydrogen to carbon dioxide. As a consequence of the removal of hydrogen from water there is formed a radical, more oxidized than water, which, in the green plant breaks down with the evolution of oxygen and the formation of water, but in the bacteria is reduced by the externally provided hydrogen donor as in the following equations:—

common primary photochemical reaction

$$4H_2O+2A+4X \rightarrow 2AH_2+4(XOH)$$

common reaction with carbon dioxide

$$CO_2+2AH_2 \rightarrow (CH_2O)+H_2O+2A$$

reductive reaction: green plant $4(XOH)+2H_2O \rightarrow 4H_2O+4X+O_2$

or bacteria $4(XOH)+2H_2S \rightarrow 4H_2O+4X+2S$

PHOTOREDUCTION AND CHEMOSYNTHESIS IN THE GREEN PLANT

Gaffron (1944) showed that under certain conditions the green alga *Scenedesmus* shows a photosynthetic reduction of carbon dioxide using hydrogen gas, thus performing the same reaction as do some purple photosynthetic bacteria. This 'photoreduction', as Gaffron called it, is only developed after the alga has been in anaerobic conditions for two or more hours (i.e. after 'adaptation'). In the dark the alga then absorbs hydrogen from an atmosphere of high hydrogen content or evolves hydrogen if the external concentration of hydrogen is small; in the light these reactions are accelerated but if carbon dioxide is present it is reduced. In the dark in the presence of oxygen and hydrogen both gases may be consumed and the energy of the oxy-hydrogen reaction coupled to the reduction of carbon dioxide. This represents a chemosynthetic reaction such as is found in *Bacillus pycnoticus*. At high light intensities and with carbon dioxide available the cells deadapt, i.e. lose their capacity for photoreduction and revert to normal photosynthesis. Deadaptation is prevented in the presence of hydroxylamine or *o*-phenanthroline, both of which have been presumed to inhibit the enzyme concerned in oxygen liberation in photosynthesis, and thus it seems probable that deadaptation occurs simply as a result of the production of oxygen or excess of the precursor of oxygen. The ability to adapt to photoreduction has now been shown to be present in a wide range of algae representing many different families. In contrast with Athiorhodaceae, hydrogen production in *Scenedesmus* is not affected by the presence of nitrogen.

Growth of *Scenedesmus* cannot be maintained by photoreduction or the oxy-hydrogen reaction; their importance lies in emphasizing the close relationship between the photosynthetic system in these plants and that in the bacteria.

THE GENERALIZED NATURE OF CARBON DIOXIDE REDUCTION

Some of the Athiorhodaceae, as mentioned earlier, are able to grow in the dark heterotrophically provided oxygen is present, oxidation supplying the necessary energy for growth. In the dark under anaerobic conditions *Rhodospirillum rubrum* shows the normal propionic acid fermentation of glucose; under aerobic conditions in the dark or anaerobic conditions in light a compound such as acetate is incorporated into the cells. Experiments with labelled acetate show that the products in light and darkness are essentially the same although the rate of formation is slower in the dark.

When carbon dioxide is supplied as the sole carbon source in the light (in the presence of molecular hydrogen) the products of fixation are different from those with acetate and are similar to the products found with green algae. The nature of these substances we shall discuss in the next chapter (Chapter 6). Thus Kamen has suggested that light in itself is not concerned with the process of carbon fixation but only modifies a pre-existing dark carbon metabolism. This is in agreement with the general view that has arisen since it has been shown that chemosynthetic and heterotrophic organisms as well as photosynthetic plants are capable of carbon dioxide assimilation. The fixation of carbon dioxide requires energy and it appears probable that the different types of organisms do not necessarily differ as to the mechanism of fixation but rather in the mechanism by which energy is supplied. The photosynthetic organisms supply energy from light via some pigment complex, the chemosynthetic from the oxidation of inorganic substances and the heterotrophs from the oxidation of organic compounds. According to the tenets of comparative biochemistry we can advance our knowledge of carbon fixation in photosynthetic organisms by studying fixation in the other groups such as the heterotrophs, and this is the subject we discuss in the next chapter.

THE CHEMOSYNTHETIC BACTERIA

Finally we return to a brief discussion of the chemoautotrophs first discovered by Winogradsky. A wide range of organisms of this type is now known which utilize the energy of oxidation of a great variety of substances for the assimilation of carbon dioxide. In addition to the 'nitrifiers' and the sulphur-oxidizing bacteria already mentioned, organisms are known which oxidize iron salts, thiocyanate, hydrogen, and carbon monoxide. Many soil bacteria are facultative heterotrophs living heterotrophically in the soil but capable of growth in purely inorganic media when hydrogen and carbon dioxide are supplied. As we shall see in the last chapter of this book it is of interest to note that in these organisms the energy produced by the oxidation of one mole of oxidizable substrate is much less than that required for the reduction of one mole of carbon dioxide. The energy liberated from the oxidation of many molecules must therefore be in some way 'pooled'. In *Nitrosomonas*, which oxidizes ammonia to nitrite, and in *Nitrobacter*, which oxidizes nitrite to nitrate, per gram molecule of carbon dioxide reduced 35 gram atoms of nitrogen are oxidized in *Nitrosomonas* whilst 101 are utilized in *Nitrobacter*. Expressed in

TABLE 5.4

TABLE OF FREE ENERGIES OF REACTIONS OF BACTERIA

	Standard free energy $-\Delta F^\circ$ per mole CO_2 or O_2 in K cal.	
Photosyntheses		
1. $\frac{1}{6}$ of $(12H_2O+6CO_2 \rightarrow C_6H_{12}O_6+6H_2O+6O_2)$		
i.e. $2H_2O+CO_2 \rightarrow (CH_2O)+H_2O+O_2$	−114·8 ($\frac{1}{6}$ of 689)	
2. $2H_2S+CO_2 \rightarrow (CH_2O)+H_2O+2S$	−17·4	
3. $2H_2+CO_2 \rightarrow (CH_2O) + H_2O$	−1·7	
	$-\Delta F^\circ$	$-\Delta F^\circ$ per mol O_2
Chemosyntheses		
1. $2H_2S+O_2 \rightarrow 2H_2O+2S$	+ 97·4	+ 97·4
2. $2S+3O_2+2H_2O \rightarrow 2H_2SO_4$	+240·6	+ 80·2
Sum 1 and 2 (2*a*). $2H_2S+4O_2 \rightarrow 2H_2SO_4$	+338·0	+ 84·5
3. $2H_2+O_2 \rightarrow 2H_2O$	+113·1	+113·1
4. $NH_4OH+\frac{3}{2}O_2 \rightarrow HNO_2+2H_2O$	+ 65·2	+ 43·5
5. $HNO_2+\frac{1}{2}O_2 \rightarrow HNO_3$	+ 18·0	+ 36·0
5*a*. $NH_4OH+2O_2 \rightarrow HNO_3+2H_2O$	+ 83·2	+ 41·6

Note: $\Delta F^\circ = -RT \ln K$, where K is the equilibrium constant of the reaction at 25°–18°, and is given for the standard state, i.e. O_2, CO_2, H_2S, H_2, as gases 1 atm.; H_2O liquid; S, (CH_2O) [i.e. $\frac{1}{6}(C_6H_{12}O_6)$] solid; NH_4OH, HNO_2, HNO_3, and H_2SO_4 as fully dissociated, each ion 1 molar. Correction to actual conditions will not very greatly affect the energy values for the oxidations. Making CO_2 0·0003 atm. would increase the energy required for 1 mol reduction by $1{\cdot}365 \times 3{\cdot}52 = 4{\cdot}8$ kg. cal.; while making O_2 0·2 atm. would decrease the energy from the oxidations by $1{\cdot}365 \times 0{\cdot}7 = 1{\cdot}0$ kg. cal. Dilution of NH_4OH and of the acids HNO_2, HNO_3 would affect both sides of the equation about equally.

terms of free energy the efficiencies of these two bacteria may then be calculated as 5·9% and 7·4%. (Note: in the calculation of efficiency no allowance has been made for the probable use of part of the energy of oxidation for metabolic processes such as growth.) Similar efficiencies of about 6% have been observed with the sulphur-oxidizing bacteria, *e.g. Thiobacillus* spp.

THE EVOLUTION OF THE PHOTOSYNTHETIC SYSTEM

Studies with bacteria have excited speculation as to the mode of evolution of the photosynthetic process. It may be supposed that in early times under the very different environmental conditions which then existed organic molecules arose spontaneously from inorganic. Eventually a self-reproducing unit was formed which was capable of utilizing the oxidation of certain inorganic compounds for the further production of organic compounds. A pigment system was later developed which allowed of the utilization of solar energy but in the first place the cells were still dependent on the supply of suitable inorganic compounds as hydrogen donors. Finally in green plants a mechanism was developed in which water was the sole hydrogen donor thus attaining a further stage in independence of the external environment.

Some evidence in support of these ideas may be obtainable from the investigation of mutants. Thus by the use of ultra-violet irradiation mutant strains have been obtained from the obligate autotroph *Thiobacillus thiooxidans* which require for growth the addition of growth factors. The necessary factors are probably one or more of the vitamin B group but the exact requirement could not be established before the strain mutated back to a type not requiring organic growth factors. Thus we have an example of the transition from a heterotroph to an autotroph in a relatively simple step. According to these views, which were developed by van Niel (1949) from the ideas of Oparin and others, the evolution of the photosynthetic system has developed step by step by the acquisition of a more complex mechanism attaining at each stage a greater independence of external reducing substances.

CHAPTER 6

MECHANISMS OF THE FIXATION AND REDUCTION OF CARBON DIOXIDE

HISTORICAL

During the earlier studies of photosynthesis carbon dioxide had been considered as a relatively unreactive molecule. From the purely chemical standpoint there were, for example, three well-known reactions: the formation of carboxylic acids from phenols (which proceeds under relatively mild conditions with phloroglucinol), the reduction to formaldehyde by magnesium metal in acid solution (Fenton), and the ready reaction of certain organic derivatives of magnesium (Grignard reagents) with carbon dioxide to give a carboxylic acid. Such chemical knowledge combined with the fact established by Willstätter that chlorophyll was an organic compound of magnesium gave plausibility to the view that the first attack on the CO_2 molecule by the green plant was through a photochemical reaction directly with chlorophyll. Although Willstätter and Stoll were unable to find with pure chlorophyll preparations evidence of any specific photochemical reactions with CO_2 the idea of an intimate relation between chlorophyll and CO_2 in the plant held an important place for many years. At this moment there is a tendency to the extreme opposite view, namely that chlorophyll and the light reaction have no *direct* relation to the uptake of CO_2 in photosynthesis. The main reasons for this change of outlook are, firstly, that the photochemical step in photosynthesis is to be regarded as a process of hydrogen transfer in which a molecule such as water participates, the reduction of carbon dioxide being an indirect process; and, secondly, that carbon dioxide must be regarded as a normal metabolite for perhaps the majority of living organisms.

Structural formulae of some coenzymes

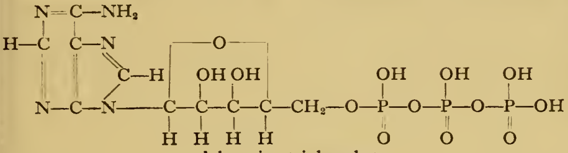
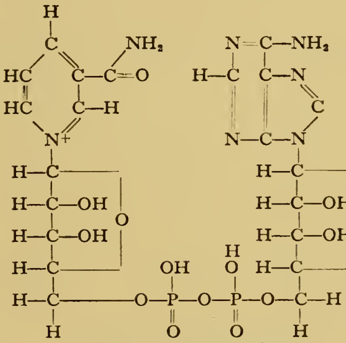
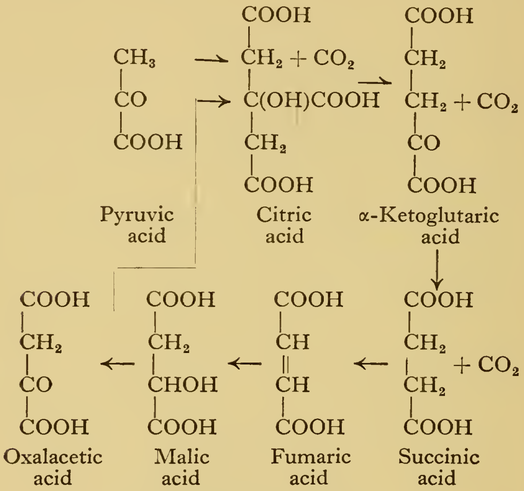
Formula	*Abbreviation*
 Adenosine triphosphate	ATP
Adenosine diphosphate (ATP minus one phosphate group)	ADP
 Diphosphopyridine nucleotide (Coenzyme I)	DPN^+ (oxidized) DPNH (reduced)
Triphosphopyridine nucleotide (Coenzyme II)	TPN^+ TPNH

FIG. 6.1

Carbon dioxide as a cellular metabolite

In the fermentation of sugar, when ethanol is formed, carbon dioxide is liberated and the cell would appear to have no further 'use' for it. On the other hand it had long been known that some heterotrophic organisms actually require a definite concentration of CO_2 for their growth. Also it had been found that certain tissue preparations from animals required the presence of bicarbonate in order to give a high rate of respiration *in vitro*. The explanation of these facts came as the result of a detailed analysis of the actual substrates concerned in the respiration of animal tissues. Szent Gyorgyi showed that the four carbon dicarboxylic acids were essential catalysts in the aerobic respiration of animal tissue; Krebs established the presence of the 'tricarboxylic cycle' and was able to complete the analysis of the respiration of carbohydrate to CO_2 and water via the formation of pyruvate. The pyruvate is derived from the carbohydrate as the result of fission of hexose into two three carbon compounds. The oxidation of pyruvate via the Krebs cycle is shown in Fig. 6.2 and it is clearly dependent on the presence of oxalacetic acid. In organisms where the

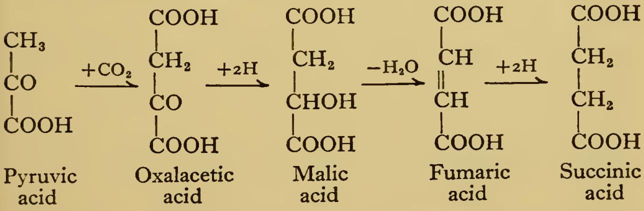


Krebs tricarboxylic acid cycle

FIG. 6.2

oxidation of carbohydrate or of acetate to carbon dioxide and water occurs via the Krebs cycle the fixation of CO_2 in pyruvic acid to form oxalacetic acid is a definite step in respiration. If no alternative path is available then CO_2 becomes essential once the supply of four carbon acids becomes depleted. The complete oxidation of pyruvate and acetate depends on the formation of acetyl coenzyme A which then reacts with oxalacetate to give citrate. Acetate itself will only give acetyl CoA at the expense of an active phosphate transferring system involving, for example, ATP. Pyruvate gives acetyl CoA directly by oxidative carboxylation, involving the simultaneous reduction of DPN, independently of an additional energy source.

The fixation of CO_2 by a heterotrophic organism was discovered by Wood and Werkman in the propionic bacteria. They later found that during the fermentation of glycerol CO_2 was utilized for the formation of succinic acid (see, for example, the review by Wood (1946)). Elsden showed independently that the rate of formation of succinate by *B. coli* in presence of pyruvate depended on the pressure of CO_2; the CO_2 presumably appearing in the succinate by the following reaction sequence:



The first step, the incorporation of carbon dioxide into pyruvic acid to form oxalacetic acid, is often referred to as the Wood and Werkman reaction.

The CO_2 fixed in a cellular metabolite in this way might well be expected to find its way by synthetic side reactions into a variety of cellular constituents. This has been shown in animal tissues using tracer CO_2: the glycogen in liver contains carbon derived from CO_2. When such experiments are made and the glycogen isolated it is found that the hexose units are labelled in the 3- and 4-positions. This suggests that the hexose is formed from a three carbon acid labelled in the carboxyl group by a reversal of the process of glycolysis. In such a scheme a key substance would be 3-phosphoglyceric acid labelled in the carboxyl group.

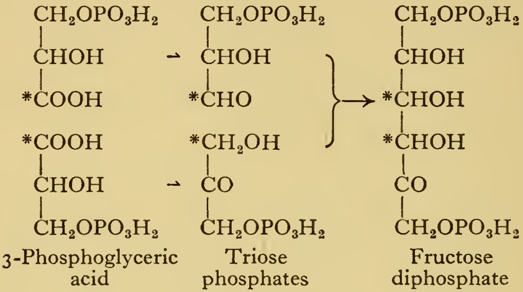


FIG. 6.3

While the basic knowledge of CO_2 metabolism has been mainly derived from heterotrophic organisms (animal tissues and micro-organisms) the same processes are presumed to hold in the respiration of green plants. For example Millerd and Bonner (1953) have shown with *in vitro* preparations from tissues of *Phaseolus* that all the component acids of the carboxylic cycle can be oxidized.

In summary, assimilation of CO_2 has been shown in the dark with representatives of all the main groups of living organisms including plants. In both plants and animals the carbon is found to appear to some extent in the carbohydrates. The isotope experiments show that the carbon is first incorporated in the 3- and 4-positions of the hexose molecule. This suggests that the process of respiration is partly reversible; the assumption being that a two-carbon compound is carboxylated and converted into triose phosphate via 3-phosphoglyceric acid.

While *a priori* there might seem to be no necessity to relate this dark fixation of CO_2 to the process of photosynthesis, the results obtained by Calvin *et al.* for photosynthesis in presence of tracer carbon have shown the rapid appearance of labelled carbon in compounds previously known to be concerned in respiration. The dark fixation of CO_2 was found by Calvin to be greatly increased in a green plant immediately following illumination. As yet we do not know whether there exists a single mechanism for the fixation of carbon dioxide specifically associated with photosynthesis; all our present knowledge of the enzyme systems concerned with the metabolism of carbon dioxide refers to the dark processes. The study of these systems in the green cell may form a significant contribution to the biochemistry of photosynthesis.

ENZYME SYSTEMS INVOLVING CO_2

Carbonic anhydrase. This enzyme catalyses the reaction between CO_2 and water to give bicarbonate ions:

$$CO_2+H_2O \rightarrow H^++HCO_3^-$$

It was discovered in mammalian blood by Meldrum and Roughton and shown to be a zinc protein compound by Keilin and Mann. Carbonic anhydrase activity has been reported to be widely present in green plants but the enzyme has not actually been isolated therefrom. It appears unlikely to be a rate determining factor in photosynthesis. The functional group of the enzyme seems to depend on the zinc atom. The enzyme is the only zinc protein compound of this type and the only metal enzyme known which reacts directly with CO_2. Since zinc is an essential element for plant growth, we may speculate whether there are other zinc protein compounds in the plant concerned with CO_2 metabolism. Carbonic anhydrase is inhibited by cyanide, and such a speculation has sometimes been invoked to explain the effect of cyanide upon photosynthesis.

Formic hydrogenlyase. This enzyme system catalyses the reaction between CO_2 and molecular hydrogen, or between the bicarbonate ion and H_2, to form formate:—

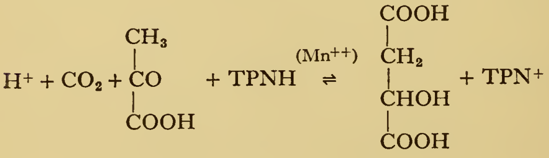
$$CO_2+H_2 \rightarrow H^++HCO_2^-$$
$$HCO_3^-+H_2 \rightarrow H.COO^-+H_2O$$

It was discovered by Stephenson and Stickland and the equilibrium constant for the reaction with the bicarbonate ion was determined by D. D. Woods. The system is freely reversible with mixtures of CO_2 and H_2 and the determination of the equilibrium constant was an important direct check upon the calculation of the free energy of formic acid from thermochemical data. Its occurrence is limited to a few groups of bacteria.

Formic dehydrogenase. Two distinct enzymes have been described, the one of bacterial origin requiring no coenzyme, the other found in seeds of many plants requiring coenzyme I. The latter system will reduce the coenzyme practically to completion for the standard oxidation-reduction potential of formate is about equal to that of the hydrogen electrode. No hydrogen however is produced because the enzyme is inert to this gas. In the case of the plant system no evidence of reversibility could be obtained either by Davison or by Vennesland (see Vennesland, 1952). Hence this plant system does not react with CO_2 to a measurable extent. The formic dehydrogenase is thus quite distinct from the formic hydrogenlyase.

The malic enzyme and the isocitric system. These are concerned in two of the best known reversible carboxylation reactions and are found to be widely present in the leaves of plants. They were originally obtained from animal tissues by Ochoa; their presence in plants was established by the work of Vennesland *et al.* and by Whatley (1951).

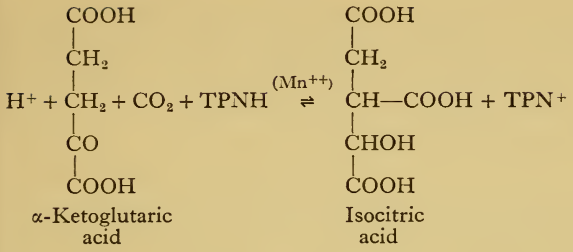
The malic enzyme catalyses the reaction



$$H^+ + CO_2 + \begin{array}{c} CH_3 \\ | \\ CO \\ | \\ COOH \end{array} + TPNH \overset{(Mn^{++})}{\rightleftharpoons} \begin{array}{c} COOH \\ | \\ CH_2 \\ | \\ CHOH \\ | \\ COOH \end{array} + TPN^+ \qquad (I)$$

Pyruvic acid Malic acid

The isocitric system catalyses the analogous reaction



$$\mathrm{H^+ + \begin{array}{c} COOH \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CO \\ | \\ COOH \end{array} + CO_2 + TPNH \overset{(Mn^{++})}{\rightleftharpoons} \begin{array}{l} COOH \\ | \\ CH_2 \\ | \\ CH\text{—}COOH \\ | \\ CHOH \\ | \\ COOH \end{array} + TPN^+} \qquad \text{(II)}$$

The activity of both systems depends on the presence of divalent metal ion: of these Mn^{++} is found at its optimum concentration (about 10^{-5}M.) to be the most effective. Mg gives a lower activity and has for its optimum effect to be present in higher concentration. In this connexion it should be noted that extreme manganese deficiency in plants results in chlorosis of the leaves; in *Chlorella* the addition of Mn^{++} causes an almost immediate increase in rates of photosynthesis and respiration.

In connexion with the role of coenzyme in these reactions it is of interest to note that Whatley has shown that the concentration of coenzyme II (TPN) is higher in leaves than in the non-green parts of many of the plant species he examined, and Vennesland has shown that DPN is present in green leaves in about the same amount.

The equilibrium constant for reaction II is given as

$$\frac{(d\text{-isocitrate}^{3-})(\mathrm{TPN}^{+})}{(\alpha\text{-ketoglutarate}^{2-})(\mathrm{CO_2})(\mathrm{TPNH})} = 1{\cdot}3 \text{ litres mole}^{-1} \ (22^\circ\,\mathrm{C.};\ \mathrm{pH}\ 7{\cdot}0)$$

where (CO_2) represents the total concentration in solution including H_2CO_3. Thus when the concentrations of oxidized and reduced coenzyme are maintained equal with a concentration of 5% carbon dioxide (i.e. $2{\cdot}23 \times 10^{-3}$M.) in the gas phase the molar concentration of *d*-isocitrate would be about 0·29% that of the ketoglutarate. Thus the equilibrium is in the direction of decarboxylation for all ordinary pressures of carbon dioxide. The same is also true for the 'malic enzyme' system. Carboxylation is observed when the ratio of reduced to oxidized coenzyme is maintained high and this can be achieved *in vitro* by adding to the reaction glucose-6-phosphate dehydrogenase in the presence of glucose-6-phosphate. The reaction is then

$$\text{Glucose-6-phosphate} + \text{TPN}^+ \rightarrow \text{6-phosphogluconic acid} + \text{TPNH} + \text{H}^+$$

followed by I or II. Carboxylation will also be favoured by removing one product of the reaction, as for example by adding aconitase in II to remove isocitric acid as citric acid, or in I adding fumarase to change malate to fumarate.

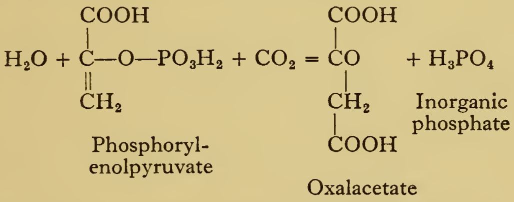
Ochoa and colleagues investigated the effect of CO_2 on the rate of fixation in malate when the hexose monophosphate system was coupled with the malic enzyme. The concentration of CO_2 in the gas phase for about half saturation was found to be 17%. Although the activity of the system is appreciable at very low CO_2 pressures the saturation of the enzyme requires a very high pressure of CO_2, that is, compared with the effect of CO_2 on the rate of photosynthesis. However, in the experiments of Elsden (loc. cit.) it was found that in the formation of succinic acid by *B. coli* the reaction appeared saturated at 5% CO_2. These facts are given here to indicate a certain complexity in the determination of whether or not a given *in vitro* enzyme system can be directly related to a physiological response.

Oxalacetate decarboxylase. The original 'Wood-Werkman reaction' (p. 89) represents a reversal of the decarboxylation of oxalacetate. In the decarboxylation reaction:

$$\text{HOOC—CH}_2\text{—CO—COOH} = \text{CH}_3\text{—CO—COOH} + \text{CO}_2 \quad (6.4)$$

the equilibrium is far over to the right. The reaction, which can occur spontaneously in solution, is markedly accelerated by the presence of a number of different metal ions; the mechanism is connected with the formation of complex ions with the metal and oxalacetate. In spite of this possibility of non-enzymic reaction, specific decarboxylases for beta keto acids are widely found in organisms. They are activated by divalent metals, particularly by Mn^{++}. Herbert (1951) obtained a crystalline preparation of an oxalacetate decarboxylase from the bacterium *Micrococcus lysodeikticus*. Attempts have been made to couple an oxalacetic decarboxylase with a malic dehydrogenase system in the presence of reduced coenzyme I, so that by starting with pyruvate, CO_2 and DPNH any oxalacetate formed would be removed by reduction to malate. The failure to produce any reaction with carbon dioxide served to show that if any oxalacetate was formed the concentration was too small to allow any reaction with the DPN-dependent malic system. Thus the decarboxylase was to be regarded as virtually irreversible. This also serves to emphasize the distinct nature of the reversible 'malic enzyme' system of Ochoa, that it is not a separable combination simply of a decarboxylase and a dehydrogenase.

Phosphoryl-enolpyruvate carboxylase. Bandurski and Greiner (1953) have described an enzyme obtained from spinach leaves which catalyses the reaction:



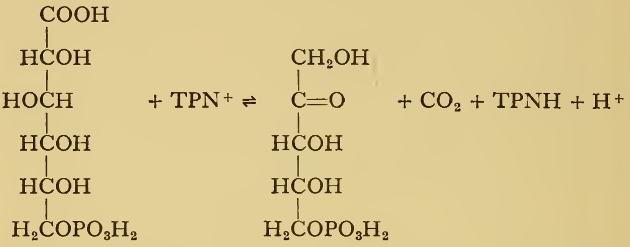
$$H_2O + \begin{array}{l} COOH \\ | \\ C\text{—}O\text{—}PO_3H_2 \\ \| \\ CH_2 \end{array} + CO_2 = \begin{array}{l} COOH \\ | \\ CO \\ | \\ CH_2 \\ | \\ COOH \end{array} + H_3PO_4 \qquad (6.5)$$

The phosphoryl-enolpyruvate can be formed from pyruvate and ATP in presence of a specific phosphokinase.

$$\begin{aligned} & HOOC\text{—}CO\text{—}CH_3 + ATP \\ & \qquad = HOOC\text{—}C(\text{—}OPO_3H_2)\text{=}CH_2 + ADP \quad (6.6) \end{aligned}$$

Hence by combining equations (6.5) and (6.6) the reversal of equation (6.4) results, together with the hydrolysis of one phosphate group from ATP giving inorganic phosphate and ADP. The incorporation of CO_2 into pyruvate may thus be accomplished by coupling with either of two processes: (1) by hydrogen transfer via the formation of malate, and (2) by phosphate group transfer via the production of phosphoryl-enolpyruvate. The significance of coupled reactions, of which this last mentioned system is an example, is discussed in Chapter 8. The emphasis here is that a mechanism for the Wood-Werkman reaction can be proposed on the basis of the plant enzyme system described by Bandurski and Greiner.

The oxidative decarboxylation of phosphogluconic acid. The formation of pentose phosphate from hexose monophosphate was described by Dickens (1938). More recently an enzyme has been purified from yeast by Horecker and Smyrniotis (1951) and which catalyses the reaction:



$$\begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{HCOH} \\ | \\ \mathrm{HOCH} \\ | \\ \mathrm{HCOH} \\ | \\ \mathrm{HCOH} \\ | \\ \mathrm{H_2COPO_3H_2} \end{array} + \mathrm{TPN^+} \rightleftharpoons \begin{array}{c} \mathrm{CH_2OH} \\ | \\ \mathrm{C{=}O} \\ | \\ \mathrm{HCOH} \\ | \\ \mathrm{HCOH} \\ | \\ \mathrm{H_2COPO_3H_2} \end{array} + \mathrm{CO_2} + \mathrm{TPNH} + \mathrm{H^+}$$

6-Phosphogluconic acid　Ribulose-5-phosphate

The system resembles the malic and isocitric enzymes in that no intermediate keto acid can be detected and that the reaction is reversible. In presence of a pentose phosphate isomerase this product is converted into an equilibrium mixture containing about 75% in the form of ribose-5-phosphate. The isomerase has been purified from a plant source by Axelrod and Jang (1953). Glock (1952) and others have shown that pentose phosphate was converted by liver extracts into hexose monophosphate to give a higher yield than could be accounted for by the three carbon residue of the pentose alone. This supported the now accepted conclusion that in the oxidation of hexose monophosphate via 6-phosphogluconic acid a cyclic process was involved. Recent work by Horecker, Racker, and others summarized by Horecker (1953) has shown that the cycle involves both pentose and sedoheptulose phosphates. It forms an important, sometimes the major, pathway for the oxidation of carbohydrate. The cycle operating with three equivalents of hexose would be summed as a reaction representing: the formation of 3 molecules of CO_2 and one molecule of triose phosphate from one molecule of hexose phosphate. The oxidation steps are the oxidation of glucose 6-phosphate (described later) and the oxidative decarboxylation of 6-phosphogluconate, TPN being involved in both steps. The sedoheptulose 7-phosphate is formed in the 'transketolase' reaction when ribulose 5-phosphate is split to give triose phosphate and a two-carbon fragment combined with the enzyme. Transketolase has been obtained in crystalline form from yeast and in purified form from spinach leaves. The coenzyme, thiamine pyrophosphate (cocarboxylase), may be separated reversibly. No reaction takes place unless an aldose acceptor, such as ribose-5-phosphate, for the C_2 fragment is present; the C_7 compound is then formed. Sedoheptulose 7-phosphate is broken down in an analogous 'transaldolase' reaction where a 4-carbon (erythrose) fragment may be split off. Ribulose phosphate acting as acceptor for the aldose fragment results in the formation of fructose 6-phosphate and triose phosphate. Starting with 3 equivalents of phosphogluconic acid or hexose monophosphate, the process may be represented as follows:

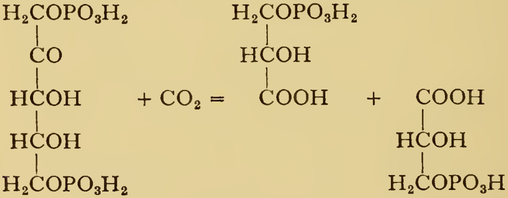
$$\begin{array}{l}
\begin{array}{rcl} 3C_6 &=& 3C_5 + 3CO_2 \end{array} \quad \text{oxidation} \\
\left.\begin{array}{rcl} C_5 &=& (C_2) + C_3 \\ C_5 + (C_2) &=& C_7 \end{array}\right\} \text{transketolase} \\
\left.\begin{array}{rcl} C_7 &=& (C_4) + C_3 \\ C_5 + (C_4) &=& C_6 + C_3 \end{array}\right\} \text{transaldolase} \\
\begin{array}{rcl} 2C_3 &=& C_6 \end{array} \quad \text{aldolase} \\
\hline
\begin{array}{rcl} C_6 &=& C_3 + 3CO_2 \end{array}
\end{array}$$

The (C_2) and (C_4) carbon residues are indicated only in the form of their complex with an enzyme, the (C_2) is most probably attached via the thiamine pyrophosphate group. This scheme, given here in a simplified form, shows the possible conversions of the sugar phosphate esters and will be referred to later (p. 109) in connexion with the interpretation of results obtained on photosynthesis with tracer carbon. There is no indication from these that a direct reversal of the part of the hexose monophosphate cycle concerned with CO_2 is important in photosynthesis; an early formation of the 1-labelled hexose would result as against the 3- and 4-labelling actually found. The transketolase and transaldolase reactions, however, are of fundamental importance in Calvin's interpretation of the formation of phosphoglyceric acid. As these reactions may be considered reversible, the following scheme may be set out:

$$\begin{array}{ll}
\left.\begin{array}{rcl} C_6 + C_3 &=& (C_4) + C_5 \\ C_3 + (C_4) &=& C_7 \end{array}\right\} & \text{transaldolase} \\
\left.\begin{array}{rcl} C_7 &=& (C_2) + C_5 \\ C_3 + (C_2) &=& C_5 \end{array}\right\} & \text{transketolase} \\
\hline
\begin{array}{rcl} 2C_6 + C_3 &=& 3C_5 \end{array} &
\end{array}$$

This represents the formation of 3 equivalents of ribose-5-phosphate from two equivalents of hexose phosphate and one of triose phosphate.

The carboxylation of ribulose diphosphate. The reaction of CO_2 with the pentulose 1-5-diphosphate to give two molecules of phosphoglyceric acid was first postulated by Calvin for the interpretation of the tracer carbon results.



$$\begin{array}{c} H_2COPO_3H_2 \\ | \\ CO \\ | \\ HCOH \\ | \\ HCOH \\ | \\ H_2COPO_3H_2 \end{array} + CO_2 = \begin{array}{c} H_2COPO_3H_2 \\ | \\ HCOH \\ | \\ COOH \\ \\ \\ \\ \\ \end{array} + \begin{array}{c} \\ \\ \\ \\ COOH \\ | \\ HCOH \\ | \\ H_2COPO_3H \end{array}$$

In recent work, developed from the results of Fager (1952), Calvin (1954) has obtained an enzyme preparation from green cell extract which produces phosphoglyceric acid from CO_2 in the presence of ribulose diphosphate. If this enzyme system can be purified and a phosphokinase isolated which will convert ribulose monophosphate into the diphosphate, then it may be that all the enzymes necessary for the formation of carboxyl-labelled phosphoglyceric acid from hexose and labelled CO_2 would be known.

Glutamic acid decarboxylase. This enzyme is widely distributed in plants and has been found both in green and in non-green tissues. Pyridoxal phosphate is required as a coenzyme, the reaction catalysed being,

$$\mathrm{COO^-.CHNH_3{}^+.CH_2.CH_2.COO^- + H^+ \rightarrow CO_2 + CH_2HN_3{}^+CH_2.CH_2.COO^-}$$

Glutamate γ-Aminobutyrate

The product of decarboxylation, γ-aminobutyric acid, has been shown to be present in extracts from a variety of plant tissues. The glutamic decarboxylase from *Clostridium welchii* has been shown to cause an incorporation of $^{14}CO_2$ into a carboxyl group of glutamic acid by Mandeles and Hanke. Reversibility has not been studied in the plant.

Decarboxylation of pyruvate by carboxylase. The formation of CO_2 from pyruvate in alcoholic fermentation is catalysed by the enzyme carboxylase which was discovered by Neuberg; the decarboxylated product acetaldehyde is then reduced to ethanol. There is no evidence that this decarboxylation is reversible. Auhagen, however, discovered that carboxylase could be resolved into cocarboxylase and a protein component (apocarboxylase). Cocarboxylase was shown by Lohman and Schuster to be the pyrophosphate of vitamin B_1 (thiamine) and the work of Peters and colleagues showed that cocarboxylase was essential for the oxidative metabolism of pyruvic acid in the animal. This coenzyme is also required for the oxidative decarboxylation of alpha-ketoglutaric acid. It has been proved that vitamin B_1 is essential for the growth of plant embryos when they are deprived of their cotyledons and supplied with carbohydrate.

The oxidative decarboxylation of pyruvate. The simplest equation for this process would be:

$$\mathrm{CH_3—CO—COO^- + 2H_2O - 2H \rightarrow CH_3—COO^- + HCO_3{}^- + H^+}$$

Pyruvate Acetate

It was found however that the formation of acetyl choline by tissue preparations from brain was dependent on the oxidation of pyruvate, suggesting that the initial product from pyruvate was not acetic acid but some other acetyl compound. Lipmann had discovered previously that pyruvic acid could be broken down by a bacterial system to yield acetyl phosphate. He also discovered that as well as cocarboxylase another coenzyme was necessary for the acetylation process: this was called coenzyme A. Later it became clear that the initial product of the oxidative decarboxylation of pyruvate must be an acetyl compound of coenzyme A. Hence the process had to be written

$$\underset{\text{Pyruvate}}{CH_3COCOO^-} + CoA + 2H_2O - 2H \rightarrow \underset{\text{acetyl CoA}}{CH_3COO\text{—}CoA} + HCO_3^- + H^+$$

Coenzyme A is then a substance concerned in group transfer (acetyl group) in a way analogous to hydrogen transport by coenzymes I and II.

Ochoa has shown how the oxidative decarboxylation of pyruvate can lead to the synthesis of citric acid. He has isolated a condensing enzyme, in a crystalline state, which catalyses the reaction:—

$$\text{Acetyl CoA} + \text{oxalacetate} \rightarrow \text{citrate} + \text{CoA}$$

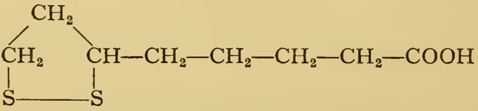
Stadtman was able to separate an acetyl transferring enzyme which catalysed the reaction

$$\text{Acetyl phosphate} + \text{CoA} = \text{acetyl CoA} + \text{inorganic phosphate}$$

The total reaction measured was:—

$$\text{Acetyl phosphate} + \text{oxalacetate} = \text{citrate} + \text{inorganic phosphate}$$

O'Kane and Gunsalus (1948) discovered that a strain of *Streptococcus faecalis* required an unknown factor connected with the oxidation of pyruvate. The factor was found to be a sulphur-containing fatty acid, a disulphide of *n*-octanoic acid, called lipoic acid. This factor appears to have a wide



α-Lipoic acid (Thioctic acid)

distribution in organisms and is significantly present in green plants. As the pyruvate oxidation system transfers acetyl to coenzyme A and hydrogen to coenzyme I, it is possible that the lipoic acid is concerned in this process by virtue of the chemical properties of the disulphide group. The oxidative decarboxylation of pyruvate has been found to depend upon the presence of four coenzymes. Three of these are sulphur compounds, namely CoA, cocarboxylase, and lipoic acid.

A reversal of the reactions of the Krebs cycle and the pyruvic system could theoretically lead to the fixation of carbon dioxide in phosphoglyceric acid. In the plant, however, such a process is considered improbable. The tracer carbon, in the experiments of Calvin, was found to be only slowly incorporated in the components of the tricarboxylic acid cycle. The significance of the oxidation of pyruvate is in connexion with the synthesis of cellular constituents from carbohydrate. The synthetic reactions are due to the group-transferring property of coenzyme A. For example, the oxidative decarboxylation of alpha-ketoglutarate can lead to succinyl CoA, which seems likely to be important in the synthesis of the tetrapyrrolic pigments (p. 39 and Fig. 3.6).

REDUCTION OF THE CARBOXYL GROUP

The change in free energy in the reduction of a carboxylic acid to an aldehyde is larger than in any of the other reduction steps that might be supposed to occur during the process of reduction of carbon dioxide to sugar. For example, the free energy of oxidation of acetaldehyde to acetic acid in dilute aqueous solution is considerably greater than that for the oxidation of formic acid to CO_2 and water. Thus hydrogen gas at atmospheric pressure even with a suitable catalyst could not appreciably reduce acetate to aldehyde. The aldehyde group is not ionized to any extent in aqueous solution while at a neutral pH the carboxyl group is regarded as completely ionized. Thus in any process of direct reduction of the carboxyl group under physiological conditions the energy of ionization is additional to the actual reducing process. These two processes may be separated so that the reduction takes place in a second step. For example, it has been found more easy to reduce a carboxylic ester than the free acid: the formation of the ester eliminates the ionization of the carboxyl group. This is the principle of a well-known reversible biological system, where the oxidation and reduction of an aldehyde/acid system is essentially a two-stage process.

The triose phosphate dehydrogenase. This enzyme catalyses the first of two steps in the oxidation of an aldehyde to a carboxylic acid; the reaction being as follows:

Triose phosphate+DPN$^+$+phosphate → diphosphoglyceric acid+DPNH

Diphosphoglyceric+ADP → phosphoglyceric+ATP

In the second step the free phosphoglyceric acid is formed by the action of a phosphokinase which transfers the phosphate group attached to the carboxyl group to adenylphosphate or to adenylic acid. Both reactions are freely reversible. They are concerned with the breakdown of carbohydrate in both fermentation and in respiration of carbohydrate via pyruvate. The triose phosphate dehydrogenase is inhibited by iodoacetate, and it is considered that the activity of the enzyme depends upon the presence of an SH group. Racker and others have considered that the specific sulphur atom belongs to glutathione which is combined with the enzyme. The SH group is regarded as being in combination with the coenzyme, DPN$^+$, in the form of a neutral DPN–S–compound. The glyceraldehyde phosphate, represented by R.CHO reacts by displacing DPNH and forming a R.CO–S–compound (which may be compared with acetyl coenzyme A). The acyl thio-link is then broken by a simultaneous reaction with more DPN$^+$ and inorganic phosphate, $HOPO_3H_2$, giving $R.COOPO_3H_2$, the original DPN–S–compound and one H$^+$. This is in accord with the fact that the H of the aldehyde group is actually transferred to the bulk concentration of DPN$^+$ forming DPNH. This mechanism shows how an oxidation-reduction step involving two equivalents of hydrogen may virtually take place in stages. A triose phosphate system has been found in the green leaves of several plant species by Gibbs (1952) and by Arnon (1952). With the plant preparations both DPN and TPN are active. This system may provide one of the main reduction steps in photosynthesis, where phosphoglyceric acid is concerned. The simultaneous discovery by Needham and Pillai and by Meyerhof and colleagues that phosphorylation of adenylic acid by inorganic phosphate was coupled to the oxidation of triose phosphate formed the beginning of a new epoch in our appreciation of the utilization of chemical energy in cellular metabolism; further detailed knowledge of the triose phosphate system is due to the work of Warburg and Christian (1939). The general ideas of the relation between respiration and the utilization of the chemical energy for synthetic processes we owe mainly to the work of Lipmann (1941). The process of phosphate group transfer has as much relevance to cellular energetics as hydrogen transfer. The two processes are intimately connected in aerobic respiration and in glycolysis.

The reduction of phosphoglyceric acid to triose phosphate can occur with reduced DPN when there is a source of ATP for the phosphate transferring step. Thus it can be seen how both H transfer and group transfers involving phosphate are important in mechanisms concerned with the reduction of CO_2 to the level of carbohydrate. Many experimental studies have been directed towards finding out whether phosphorylation reactions are to be regarded as part of the photosynthetic mechanism. The evidence in favour is strong; it is, however, circumstantial because so far we have no knowledge of a cell-free system involving phosphate which can be directly related to the process of photosynthesis.

Hexose and hexose phosphate dehydrogenases. The glucose 6-phosphate dehydrogenase has been shown to catalyse the reaction:

$$\text{Glucose-6-phosphate} + \text{TPN}^+ = \text{6-phosphogluconate} + \text{TPNH} + \text{H}^+$$

The enzyme is found to be present in widely different types of organisms, including plants. The reaction would be the first step in the oxidative breakdown of hexose by the transaldolase transketolase cycle (p. 97). As written the reaction is virtually irreversible. It has, however, been proved that in the case of the glucose dehydrogenase the initial product is gluconolactone. Korkes and Strecker were able to show that the reaction is reversible under acid conditions where hydrolysis of the lactone is diminished. We may also conclude from the work of O. Cori and Lipmann that the oxidation product of glucose 6-phosphate is also the lactone. Thus, as in the case of the triose phosphate system, the formation of the carboxylic acid from the aldose can be regarded as a two-step process. The hexonolactones, however, have not been shown to take part in any group transfer reaction, and would appear to hydrolyse spontaneously.

REDUCTION OF CARBONYL AND ALCOHOL GROUPS

When related to the production of oxygen from water the reduction of aldehydes and ketones to alcohols involves less energy than the previously described reductions; in the presence of reduced coenzyme I the reduction can proceed, resulting in a fairly complete oxidation of the coenzyme when equilibrium is reached. The ethanol-acetaldehyde system is of this type. When ethanol is formed by dismutation of acetaldehyde with the production of acetate the reaction is to be regarded as virtually irreversible. Again the further reduction of the alcohol to the hydrocarbon level also is by no means the largest energy step in CO_2 reduction. This may be seen from the fact that methane is produced by bacteria from CO_2 plus a carbon compound such as formate, the process actually yielding energy, which is utilized for growth. Oxidations and reductions which involve CH_2, CHOH, and CO groups are fundamental in fermentation and glycolysis, and thus are represented in all living cells.

THE PRODUCTS OF PHOTOSYNTHESIS

During growth the immediate products of photosynthesis are converted by a series of metabolic reactions into a variety of compounds including carbohydrates, fats, and proteins. In short, the final product of photosynthesis is the plant itself. In the first chapter we described the determination of photosynthesis by the appearance of starch. Smith

(1949) was able to show with the sunflower leaf that during active photosynthesis the accumulated starch will account for nearly the whole of the carbon assimilated. The mature leaf might perhaps be regarded as a tissue with little growth. In Chapter 1 the over-all response to light was formulated simply in terms of carbohydrate formed. But this simple formulation is never strictly true because proteins as well as carbohydrates are to a greater or lesser extent concerned in the metabolism of leaves. In sunflowers the assimilatory quotient CO_2/O_2 is very nearly unity, corresponding to the formation of carbohydrate. In wheat leaves, on the other hand, the assimilatory quotient is sensibly less than unity, the difference being accounted for by protein synthesis. The earlier studies of the carbohydrate relations in leaves suggested that a soluble carbohydrate, either hexose or sucrose, was the first recognizable product of photosynthesis to accumulate. This again justified the simple formulation of the over-all process as resulting in formation of $(CH_2O)_n$.

The photosynthetic studies with micro-organisms have emphasized the need to formulate, as the end product of the process, the cell substance as a whole, the organism itself. Here the nature of the final product is best defined by the value of the photosynthetic quotient and the analyses of total cell material rather than by any precise chemical formula. Thus the chemical nature of what might be termed the first product of photosynthesis may have no relation to the measured photosynthetic quotient; the adjustment to the carbohydrate level or to those corresponding with amino acids might be regarded as secondary processes. These latter would of course be included in the over-all measurement of the photosynthetic quotient.

When organisms are supplied with nitrate as the source of inorganic nitrogen the value of the photosynthetic quotient does not only indicate the extent of the reduction of the CO_2. Nitrate has to be reduced as well when proteins are formed, and part of the oxygen measured may correspond to the reduction of nitrate. Such an effect, a lowering of the photosynthetic quotient due to nitrate reduction, was discovered by Warburg (1922) with *Chlorella*. Although the reduction of nitrate to the level of ammonia has been shown to occur in the dark, the rapid reduction of nitrate observed in illuminated green tissue or cells seems to depend upon the photosynthetic activity. This may be because one of the intermediary products of photosynthesis is utilized specifically for the reduction of nitrate, in addition to the process occurring in most plants independently of light. Indeed, Gorham found that with one strain of *Lemna* reduction of nitrate was only possible in the light. Since in the plant sulphur is absorbed as sulphate, reduction to the level of H_2S (or nearly so) is an essential step in protein synthesis; there is however no evidence that this reduction is dependent upon light.

Generally, it is best to regard as the product of photosynthesis the whole of the addition to the cell in carbon compounds which takes place during the period of illumination. Thus in a sunflower leaf the first starch to be formed in the presence of tracer CO_2 may have no labelled carbon, yet for a physiological description the starch formed may be considered a product of photosynthesis.

At the present time a definition of a first product of photosynthesis is that of a stable compound in which the carbon atom derived from the CO_2 is first found. The studies made with tracer CO_2 have rendered this line of approach far more possible than did the former methods of direct chemical analysis of the plant material exposed to different external conditions.

Experiments with radioactive carbon

Investigations of the path of carbon during photosynthesis were initiated using radioactive carbon by Ruben, Kamen, and Hassid (1940) at a time when the only tracer available was the short-lived ^{11}C. The short life of the tracer necessitated the use of brief periods of illumination of the order of minutes; nevertheless as we shall see in the light of subsequent work during such relatively brief periods the tracer carbon becomes distributed amongst many compounds. Thus it is not surprising that these workers were unable to identify any single compound in which the radioactivity became concentrated; nevertheless they found much of the tracer in the light was fixed in the carboxyl groups of α-keto acids. Subsequently the long-lived tracer ^{14}C became available and was exploited in the researches of Calvin and colleagues (1949, 1951) and Gaffron and colleagues. In many of the experiments a steady state of photosynthesis was established in the presence of $^{12}CO_2$, then $^{14}CO_2$ was added at zero time and illumination continued for a specified interval until the cells were rapidly killed by immersion in boiling alcohol. Subsequently the cell contents were analysed. Calvin and colleagues combined the tracer method with analysis by paper chromatography and radioautography and in this way succeeded in identifying many compounds into which tracer was incorporated in green cells in the light.

During periods of illumination of several minutes' duration many carbon compounds showed activity including fats and proteins. When the period of illumination was shortened to 30 seconds fewer compounds showed activity and these included alanine, aspartic acid, malic acid, pyruvic acid, and phosphoglyceric acid (PGA). In order to distinguish which of these was most intimately concerned in photosynthesis the period of illumination was decreased still further, it being argued that the shortest periods should result in tracer appearing in only one compound, the first product of photosynthesis. With periods of less than 2 seconds most of the tracer appears in PGA. Further, as the period of illumination is shortened the percentage of tracer carbon incorporated in PGA compared with the total tracer incorporated rises, extrapolating to some 75% at zero time. This probably indicates that PGA is the first intermediate formed in detectable amounts. Of the remaining 25% malic acid constitutes 3%, free glyceric acid 2%, phospho-enolpyruvic acid 3% and the rest consisting of small amounts of glucose 1-phosphate, sedoheptulose phosphate, fructose monophosphate, and dihydroxyacetonephosphate. Some of these compounds such as phospho-pyruvic acid show with brief periods of illumination a very low percentage of tracer, the percentage rising as the period of illumination is lengthened but again decreasing as the period of illumination is lengthened still further. These compounds may be considered as formed second in a catenary sequence of reactions. The third group such as sucrose, representing the final products of the reaction sequence, show a progressive increase in tracer from the shortest to the longest periods. Thus it appears that PGA is the first photosynthetic intermediate which during further illumination is in part transformed to compounds of the second class and then finally to ultimate products.

In other experiments a different procedure was used, the cells being first illuminated in absence of carbon dioxide, darkened and tracer added at the same instant, and then killed and analysed after a known period of darkness. This 'pre-illumination' resulted in a marked increase in tracer uptake during the dark. Subsequent to cessation of illumination this increased ability for fixation of tracer decays with a half time of the order of one minute. Tracer fixed after 'pre-illumination' is mainly confined to PGA and phosphopyruvic acid in spite of the relatively long times during which fixation occurs. These experiments are interpreted as showing that fixation after 'pre-illumination' represents the combination of carbon dioxide with acceptor molecules generated in the light and persisting in the dark; further reaction of the carbon dioxide acceptor compound does not occur in the dark.

Initially this acid is labelled only in the carboxyl group; the hexose derivatives first formed being predominantly labelled in the 3- and 4-positions (the central carbon atoms). This suggests that carbon dioxide is added to some two-carbon precursor forming a new carboxyl group and that two molecules of the three-carbon acid so formed condense, the two carboxyl ends joining together, to give the hexose molecule (p. 90). During longer periods of photosynthesis the labelling of all the carbon atoms in the three-carbon acid tends to equality; similarly with the hexose formed. Thus the hypothetical two-carbon precursor must also be derived from the carbon dioxide assimilated. For this reason Calvin postulated that part of the phosphoglyceric acid presumed to be formed would give rise to hexose and carbohydrate while part would be utilized in a process providing the two-carbon atom precursor.

Various suggestions as to the nature of the two-carbon compound were made in attempts to include those reactions known to result in carbon dioxide fixation in heterotrophs, such as the Wood-Werkman reaction. The evidence now indicates that only one port of entry for carbon dioxide is probable and that this results in the formation of PGA. Suggestions as to the nature of this reaction have been modified by the recent finding that among the compounds which become radioactive during very brief periods of illumination are sedoheptulose phosphate and ribulose phosphate. After 5 seconds illumination these compounds are respectively labelled in their 3, 4, 5, and 3 with some but less in 4 and 5 C atoms. This is consistent with the following sequence of reactions.

$$3C_5 + 3CO_2 \longrightarrow 6\ \text{PGA}\ (C_1C_2C_3{*})$$

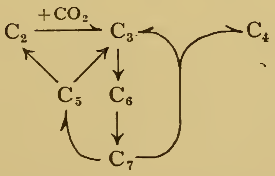
$$6\ \text{PGA} \xrightarrow{\text{light}} 6\ \text{triose phosphate}$$

$$2\ \text{triose phosphate} \longrightarrow \text{Hexose}\ (C_1C_2C_3{*}C_4{*}C_5C_6)$$

$$\underset{(C_1C_2C_3{*}C_4{*}C_5C_6)}{\text{Hexose}} + \underset{(C_1C_2C_3{*}C_3{*}C_2C_1)}{2\ \text{triose}} \longrightarrow \underset{(C_1C_2C_3{*}C_4C_5)}{\text{Pentose}} + \underset{(C_1C_2C_3{*}C_4{*}C_5{*}C_6C_7)}{\text{Sedoheptulose}}$$

$$\text{Sedoheptulose} + \text{Triose} \longrightarrow \underset{(C_1C_2C_3{*}C_4C_5\ +\ C_1C_2C_3{*}C_4{*}C_5{*})}{2\ \text{Pentose}}$$

The reactions can be illustrated by the following diagram:



Further evidence for this mechanism has been obtained by suddenly decreasing the concentration of carbon dioxide when the labelling of the 5 C compound increases while that of the 3 C decreases proportionately. Also immediately after the light is turned off there is a marked decrease in labelling of the 5 C compound with an increase in the 3 C compound.

The results with tracer carbon are beginning to give clear indications as to the enzyme systems likely to be concerned in the reduction of carbon dioxide to sugars. There is little evidence that incorporation of carbon dioxide occurs via reversal of the reactions of the Krebs cycle; greater importance is indicated for the enzymes concerned with 5 C rather than 4 C compounds. Calvin and Massini (1952) have suggested that during illumination lipoic acid, a co-factor for oxidative decarboxylation of pyruvic acid, is reduced and made inactive. All the work with radioactive carbon has been confined to a few species of plants, mainly *Chlorella*, *Scenedesmus*, and barley. This should perhaps suggest some hesitancy in accepting broad generalizations too readily. Nevertheless tracer carbon has already proved itself a powerful tool.

ENERGETICS OF THE PROCESSES INVOLVED IN THE REDUCTION OF CO_2

The formation of a hexose sugar from carbon dioxide (gas) and water (liquid) by the over-all reactions,

$$6CO_2+6H_2O \rightleftharpoons C_6H_{12}O_6+6O_2, \quad \Delta F^\circ=690{,}000 \text{ cals.}$$

would require a very strong reducing agent. The transfer of 24 equivalents of hydrogen is concerned. Each monovalent step therefore requires 28,800 cals. This represents a potential of –0·02 volts, a potential more reducing than the hydrogen electrode at one atmosphere pressure. In the metabolism of carbohydrates in living cells the main pathway for hydrogen transport is through the coenzymes DPN and TPN. Their characteristic potentials are about 0·1 volt more positive than the hydrogen electrode at a physiological pH (see Table 7.1). Thus they are only to be regarded as effective in hydrogen transfer at potentials less reducing than ordinary hydrogen. So far no coenzyme or redox-catalyst of a potential more reducing than the hydrogen electrode has been found in the plant. Therefore if it is accepted that the reduction of CO_2 is a dark process our present knowledge would indicate that it would have to involve more than the steps corresponding to the direct reaction with four equivalents of hydrogen. To suppose the minimum number of steps, necessitates fresh hypotheses concerning the nature of the enzyme systems concerned. A more complex model of CO_2 assimilation involving group transfer reactions as well as H transfer requires less hypotheses of a biochemical character than a simple model of direct reduction. All the energy consumed in each process must ultimately come from light when photosynthesis is represented by the over-all process. The quantum of red light concerned in the photochemistry of chlorophyll most probably corresponds to 41,500 cals. per mol. (the maximum for fluorescence of chlorophyll *a*). This is considerably greater than the 30,000 cals. required for each of the H transfer steps to CO_2 with concurrent production of molecular oxygen. From several lines of argument which have been discussed from a physical standpoint by Franck and others it would appear impossible that the whole of the energy corresponding to each quantum is consumed in some corresponding chemical process. The reduction of the coenzymes together with the oxidation of water to O_2 at about one atmosphere pressure would correspond to rather more than half the energy of one quantum for each H equivalent transferred.

From the data collected and referred to in this chapter it would appear that the reduction of one molecule of CO_2 to the state of carbohydrate by known dark processes would require a molecule of coenzyme to be reduced and oxidized more than twice which means the total transfer of more than 4 H equivalents. Four of these equivalents would be concerned with reduction and the remainder with group transfer reactions, which as was seen, enable the reduction stages to be brought with the range of the reduction-oxidation of the coenzymes. Such a hypothetical model is based on the assumption that the difference between the dark and the light assimilation of CO_2 is simply one of intensity and not of kind. This assumption could be supported by many of the results obtained by Calvin. On the other hand it has sometimes been maintained that in the normal green plant the difference between the light and dark reduction process is in some ways quite fundamental.

The energy relationships of some of the processes that have been referred to are summarized in a potential diagram (Fig. 7.2 and Table 7.1; pp. 121, 122).

The scale is in volts and refers to reactions in dilute solution at pH 7. The 'reducing action' of the light corresponding to the quantum of wavelength 675 mμ is here represented as a difference of potential from the oxygen electrode (which has a potential of = +0·8 volt at pH 7 when referred to the standard H_2 electrode at pH 0). This choice is completely arbitrary, but is directed by the fact that O_2 is the only recognizable product of photosynthesis on the oxidizing side. If for example we were to assume that H_2O_2 was the product of a light reaction then the reducing action would be less by about 0·6 volt. The important point to note in the diagram is the relative positions of the O_2, H_2, and coenzyme potentials in relation to those of some of the systems that have been discussed. Dixon (1949) has given a diagram of the free energies in terms of ΔF and rH of a number of biological systems. The hydrogen electrode supplies the point of reference. For the energy corresponding to the light process we have no standard of reference, but it is plausible to take a point somewhere between the O_2 electrode and the O_2 over-potential corresponding to the decomposition of H_2O_2 by the reaction $H_2O_2 = \frac{1}{2}O_2 + H_2O$, $\Delta F = -25{,}100$ cals. The value of a diagram of free energies is that it gives the data required to determine how far a given reaction will proceed if allowed to come to equilibrium. The diagram can assist in the formulation of hypothetical reaction schemes to guide our interpretation of experiment. The photochemical process is only to be formulated in terms of steady states, and the fact that it has not been possible to obtain equilibrium data is reflected in our inability to fix a reference point for the available light energy on the free energy or potential diagram.

CHAPTER 7

THE PRODUCTION OF OXYGEN BY PREPARATIONS OF ISOLATED CHLOROPLASTS

INTRODUCTION

THE assimilation of CO_2 requires a reduction process; it must be accompanied by a complimentary process of oxidation which in the green plant results in the production of molecular oxygen. This production of molecular oxygen is a characteristic response of the illuminated green plant to the presence of carbon dioxide. In this chapter we shall be concerned with the mechanism of the production of oxygen rather than with the mechanism of the reduction of carbon dioxide.

When the cells of the green leaf are broken it is possible to separate a fluid which contains chloroplasts and chloroplast fragments but such preparations produce only minute amounts of oxygen in the light. The early work of Haberlandt and of Ewart in the late nineteeenth century had shown that chloroplasts isolated from the leaves of a moss or *Selaginella* produced in the light an amount of oxygen so small as to be detectable only by the use of luminescent bacteria. In 1925 preparations from dried leaves were shown by Molisch also to produce small amounts of oxygen upon illumination, but this was observed only when the leaves were powdered under water and the powder left in contact with the water extract. This *in vitro* reaction with light was originally considered to represent a very feeble activity of normal photosynthesis, but this interpretation was shown to be unjustified by the work of Hill. Using chloroplast preparations from *Stellaria media* and *Lamium album*, Hill and Scarisbrick (1940) showed that the photochemical production of oxygen required the presence, not of carbon dioxide, but of a suitable hydrogen acceptor and that when this was added to the preparation oxygen could be produced in light at a rate comparable with that of the maximum rate of photosynthesis shown in the living plant.

The 'chloroplast reaction' (or 'Hill reaction') is a term used to describe the production of oxygen by isolated chloroplasts or by preparations from them. While this process does not depend directly on carbon dioxide it shares two of the characteristic features of photosynthesis: the conversion of light energy into chemical energy, and the appearance of oxygen in molecular form. The oxygen produced is stoichiometrically equivalent to the reduction of an added substance. The nature of the added substance, or hydrogen acceptor, will determine the amount of chemical energy accumulated as a result of illumination. The *in vitro* preparation may be regarded as a photocatalyst, if the term is used in a special sense, meaning here that the over-all reaction would not tend to take place spontaneously in the dark.

HYDROGEN ACCEPTORS FOR THE CHLOROPLAST REACTION

Chloroplasts suspended in an aqueous extract of acetone-treated leaves evolve oxygen in the light at a maximum pressure of the order of 1 mm. Hg. pressure. If instead of acetone extract potassium ferric oxalate is added to the illuminated chloroplast preparation as an external hydrogen acceptor, oxygen is evolved up to a pressure of 4 mm. Hg. Practically complete reduction of the ferric compound to ferrous takes place and the amount of iron reduced is in stoichiometric equivalence to the oxygen produced. The photochemical reaction may be represented by the equation:

$$4K_3Fe(C_2O_4)_3+2H_2O+4K^+ \rightarrow 4K_4Fe(C_2O_4)_3+4H^++O_2$$
$$\Delta F=+18{,}700 \text{ cals. per hydrogen atom transferred at pH 7}$$

If the re-oxidation of the ferrous oxalate is prevented by adding potassium ferricyanide, oxygen is produced even in the presence of 21% (i.e. in air). The chloroplasts of some plants, e.g. *Beta vulgaris* and *Spinacia oleracea*, will react directly with ferricyanide and the reaction is then represented by the equation:—

$$4K_3Fe(CN)_6+2H_2O+4K^+ \rightarrow 4K_4Fe(CN)_6+4H^++O_2$$
$$\Delta F=+8{,}500 \text{ cals. per hydrogen atom transferred at pH 7}$$

Alternatively benzoquinone may be used as a hydrogen acceptor, when it is quantitatively reduced to hydroquinone with the production of a corresponding amount of oxygen. This reaction was first demonstrated by Warburg and Lüttgens (1946) and with this substance they demonstrated that 100 moles of quinone per mole of chlorophyll present, or a weight of quinone equal to the total weight of the granules, could be reduced, thus finally removing the possibility that the 'chloroplast reaction' might be due to the liberation of oxygen from a limited quantity of a peroxide originally present in the chloroplast preparation.

$$2C_6H_4O_2+2H_2O \rightarrow 2C_6H_4(OH)_2+O_2$$

$\Delta F = +12{,}300$ cals. per hydrogen atom transferred at pH 7

Dyestuffs such as phenol indophenol and 2:6-dichlorophenol indophenol have also been used as hydrogen acceptors. Oxygen itself can also serve as a hydrogen acceptor in the chloroplast reaction as shown by Mehler (1952).

In all these reactions a molecule of water is involved, suggesting that the oxygen might originate from the water rather than from the hydrogen-accepting reagent. This latter point was established by Holt and French (1948) who, using water enriched with ^{18}O, showed the isotopic composition of the oxygen produced to be the same as that of the water present.

Attempts have been made to determine the smallest unit of the chloroplast which is capable of reaction. Preparation of small particles of fairly uniform size may be made by grinding the chloroplasts with powdered glass; suspensions of very small chloroplast fragments in which the grana are largely disintegrated have been obtained by the use of either ultrasonic vibrations or by forcing the chloroplast suspension under high pressure through a small opening. Even with whole chloroplast suspensions good activity is only obtained if the preparation is made at low temperature, the activity decreasing rapidly at room temperature. With each successive fragmentation the activity of the preparation decreases but some partial restoration is obtained with the smallest particles after coagulation by the addition of salts.

METHODS OF MEASURING THE CHLOROPLAST REACTION

(*a*) *Determination of the oxygen production.* The most reliable data are obtained when the production of oxygen is measured in relation to a known added amount of hydrogen acceptor. With hydrogen acceptors of more negative reducing potential the measurement of oxygen may be made spectroscopically using the blood pigment haemoglobin. The whole reaction is observed in the liquid phase and both the total amount of O_2 and the concentration of O_2 in equilibrium with the haemoglobin can be determined at any instant during the progress of the reaction. With reagents of more positive potential the direct manometric determination of the O_2 produced has been found satisfactory. The polarographic and electrode potential methods which have been successfully employed for the determination of O_2 in photosynthesis are less easy to apply to cell-free preparations. The very sensitive method for detecting O_2, depending on the quenching of the luminescence of the dye trypoflavine (an acridine derivative), has been used to determine O_2 at pressures of the order of 10^{-5} mm. Hg. This last method, developed by Franck, Pringsheim, and Lad (1945) replaced the earlier qualitative methods of great sensitivity, depending on bacterial luminescence or on bacterial motility, by a quantitative one only perhaps one order less sensitive.

(*b*) *Determination of the amount of H acceptor reduced.* When a dye is used as a hydrogen acceptor the progress of the reaction can be followed by a photometric method. The method using dichlorphenolindophenol (a blue dye sufficiently soluble in water) had been developed by French and co-workers and shown to give reliable results. The danger of this method is that the dye is liable to reduction by processes involving the direct oxidation of the cellular constituents. It is not always certain that these effects can be accounted for by a dark control. Chemical analysis can be also used with any hydrogen acceptor for the chloroplast reaction. In some cases the degree of reduction can be determined by an electrode measurement.

(*c*) *The determination of the change in* H^+ *ion concentration.* In the reaction:—

$$4Fe(CN)_6+2H_2O \rightarrow 4Fe(CN)_6+4H^++O_2$$

all three of the methods, *a*, *b*, and *c* have been applied and have been found to yield concordant results. With such a reaction as that with ferricyanide the consistency of the different methods indicates clearly the part played by water in the *in vitro* photochemical production of oxygen.

COMPARISON OF THE CHLOROPLAST REACTION AND PHOTOSYNTHESIS

If the chloroplast reaction is to be of use in the biochemical analysis of reactions taking part in photosynthesis the conditions of oxygen production *in vitro* must be considered in relation to the production of oxygen in the living cells. When the same hydrogen acceptors, which have been used with chloroplast preparations, are added to living cells, in the absence of carbon dioxide, oxygen is produced upon illumination. This evidence alone does not establish that the hydrogen acceptor is acting as a substitute for carbon dioxide because in the living cell there is the possibility of a dark reaction between the added substance and carbohydrate resulting in the production of carbon dioxide. In the light normal photosynthesis will convert such carbon dioxide into oxygen which will be evolved. Such a dark process apparently occurs when nitrate, benzaldehyde, or ferricyanide is added to *Chlorella* cells, although in the case of nitrate the rate of production of oxygen is greater in the light than that of the carbon dioxide production in the dark. Thus in this case either the oxidation reaction which occurs in the dark is accelerated in the light (owing for example to the increased concentration of some carbon intermediate which reacts with the nitrate) or there is in addition some direct photochemical reduction of nitrate accompanied by oxygen production. When *o*-benzoquinone is added to *Chlorella* cells oxygen is liberated in the light and in this case no difficulty in interpretation arises, since both respiration and normal photosynthesis are inhibited by the added quinone. Thus in the presence of quinone *Chlorella* cells resemble the non-living chloroplast preparations. In a discussion of the factors which affect the chloroplast reaction we shall therefore include observations on whole cells to which quinone has been added.

(*a*) *Inhibitors of oxygen production*

The chloroplast reaction is strongly inhibited by urethanes and by *o*-phenanthroline in about the same concentration range as is photosynthesis in the living cell. Azide and hydroxylamine also inhibit the chloroplast reaction but there is little agreement between different investigators as to the effective concentration range; this is partly due to the fact that inhibition with these poisons is progressive. Cyanide has no effect on the chloroplast reaction whereas, as we have seen, it is a strong inhibitor of photosynthesis, probably affecting the carboxylation reaction. Thus these results are in agreement with the conclusion that the mechanism of oxygen production in the chloroplast reaction is essentially the same as that in photosynthesis.

(*b*) *Light intensity*

The chloroplast reaction shows saturation within the same order of intensity as photosynthesis, suggesting that possibly there is a dark process in the chloroplast reaction which becomes limiting at about the same intensity as in photosynthesis when the carbon dioxide concentration is high. In the few experiments which have been reported there is not an exact correspondence between the light curves of photosynthesis and the chloroplast reaction. If the curves were identical it would indicate that the quantum efficiency of the two processes was the same, but up to the present, slightly lower values have been obtained for the efficiency of the chloroplast reaction. With *Chlorella* cells for low light intensities of the same energy flux the oxygen production in photosynthesis in alkaline media and in reduction of quinone are of the same order.

(*c*) *Intermittent illumination*

The effect of intermittent illumination was investigated with *Chlorella* cells in the presence of quinone, using the same type of illumination as was used in photosynthesis in the earlier experiments of Emerson and Arnold (described in Chapter 4). The results obtained were similar to those for photosynthesis and it was concluded that the chloroplast reaction must also involve a dark process whose reaction velocity is of the order of $\frac{1}{100}$ second just as was found for photosynthesis.

(*d*) *Effect of temperature*

The activity of chloroplast preparations is rapidly lost at temperatures above 45° C. This indicates a greater degree of thermo-lability than is characteristic of many enzyme systems but corresponds with the sensitivity of photosynthesis to temperature found by Blackman. The detailed comparison of the temperature characteristics of photosynthesis and of the chloroplast reaction is not straightforward because it is likely that some of the dark reactions are not common to both processes. With abundant hydrogen acceptor and at high light intensities the temperature coefficient between 10° and 20° C. is of the same order as that for photosynthesis with high concentrations of carbon dioxide. This observation together with those on the affect of intermittent illumination make it possible that the same dark reaction is limiting under these conditions in photosynthesis and in the chloroplast reaction.

(*e*) *Induction phenomena*

In most plants a lag period in photosynthesis or 'induction' effect is observed subsequent to an aerobic dark period, the half time varying from plant to plant. If the dark period is anaerobic the lag period is prolonged. With the chloroplast reaction no induction period has been observed. Even when chloroplasts were illuminated in the presence of an oxygen concentration as low as 1/100,000 mm. Hg pressure the full rate of oxygen production was observed as soon as it was possible to record the rate (within about $\frac{1}{10}$ second). In these experiments by Franck and colleagues oxygen was determined using the extremely sensitive method of fluorescence quenching. On the other hand with photosynthesizing

Chlorella cells there was a small initial production of oxygen immediately upon illumination attaining a pressure of the order of 5/100,000 mm. Hg but after this initial evolution the production of oxygen fell to a lower value to be followed in the course of some minutes by a gradual rise to a higher steady rate (see Fig. 7.1). The immediate response of the cells to light may be compared with the chloroplast reaction using natural hydrogen acceptors such as are in acetone leaf extract, and present in the cell in limited amount, the subsequent rise representing the slow development of the normal photosynthetic mechanism (Hill and Whittingham, 1953). Further work by Allen in Franck's laboratory has shown that in *Scenedesmus*, unlike *Chlorella*, the development of the photosynthetic mechanism is independent of the presence of free oxygen. Hitherto induction phenomena have been interpreted by Franck in terms of the formation and removal of natural inhibitors of the photochemical system and by Wassink as due to changes in the concentration of a suitable hydrogen acceptor for the photochemical system. More precise details are necessary before

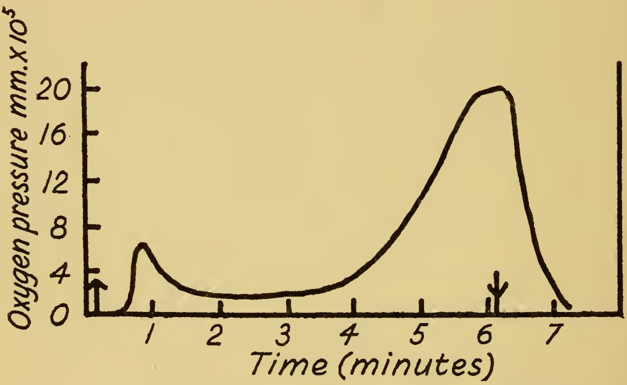


FIG. 7.1. Time course of oxygen evolution from *Chlorella* cells under anaerobic conditions illuminated at 0° C. (After Franck, Pringsheim, and Lad.)

speculation is likely to be profitable, but it may well be that a correct interpretation of the induction phase in photosynthesis might provide a guiding principle for the setting up of biochemical models of photosynthesis *in vitro*.

TABLE 7.1

STANDARD OXIDATION-REDUCTION POTENTIALS

(*a*) *Biological substances*

The following table lists the standard oxidation reduction potential E_0' at pH 7·0 and at 25° C. referred to the standard hydrogen electrode. It also gives the standard free energy (ΔF_0) for reduction in the reaction $A+H_2 \rightarrow AH_2$ per hydrogen atom or electron transferred and the free energy ($\Delta F_0'$) for the reaction $A+H_2O \rightarrow AH_2+\frac{1}{2}O_2$ (i.e. referred to a theoretical standard oxygen electrode). The relation between ΔF_0 and E_0' is

$$\Delta F_0 = -23{,}068n(E_0' + 0{\cdot}06 \text{ pH})$$

in which n has been taken as unity.

A	Reduced product	Standard		
		Potential E_0' volts	Free energy ΔF_0 Kcals.	Free energy $\Delta F_0'$ Kcals.
$\frac{1}{2}O_2$	H_2O	0·81	−28·4	0
Cytochrome *f*	Reduced cytochrome *f*	0·38	−18·4	10·0
Cytochrome *c*	Reduced cytochrome *c*	0·28	−16·1	12·3
½ Coenzyme I	½ Reduced coenzyme I	−0·32	− 2·3	26·1
H^+	$\frac{1}{2}H_2$	−0·42	0	28·4
$\frac{1}{4}CO_2$	¼ Carbohydrate ($\frac{1}{6}C_6H_{12}O_6$)	−0·44*	+ 0·4*	28·8*

* Average value based on the free energy of the reaction $\frac{1}{6}C_6H_{12}O_6+O_2 \rightarrow CO_2+H_2O, \Delta F_0'+115{,}000$ cals.

(*b*) *Other chloroplast reactants*

Ferricyanide	Ferrocyanide	0·44	−19·9	8·5
Quinone	Hydroquinone	0·28	−16·1	12·3
Phenol indo-2 : 6-dichlorphenol	Reduced dye	0·22	−15·3	13·1
Ferric potassium oxalate	Ferrous potassium oxalate	0	− 9·7	18·7

THE CHLOROPLAST REACTION IN RELATION TO HYDROGEN TRANSPORT

We have seen in Chapter 5 that van Niel suggested the generalization that the essential photochemical reaction in all photosynthetic organisms is the 'splitting' of a water molecule, the hydrogen being utilized for the reduction of carbon dioxide whilst the oxidized radical produced either breaks down with the liberation of oxygen as in the green

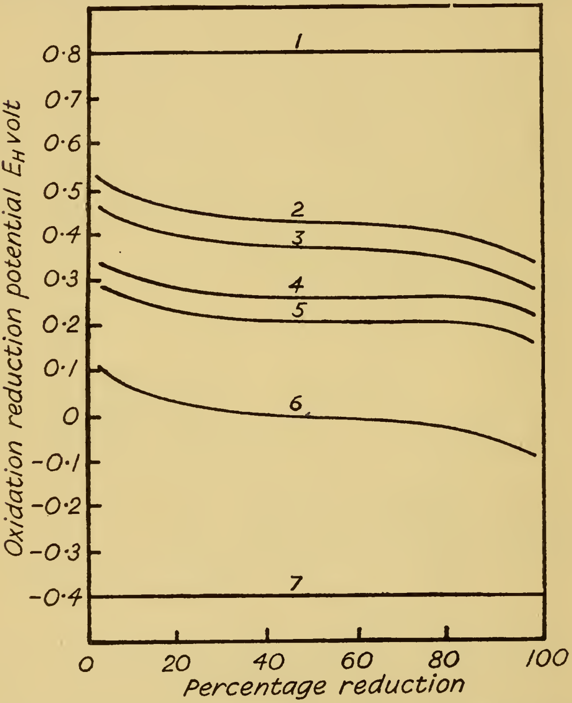


FIG. 7.2. Potentials and percentage reduction for substances concerned with the chloroplast reaction. (After R. Hill.) 1. Oxygen; 2. ferricyanide; 3. cytochrome *f*; 4. quinone; 5. 2:6-dichlorindophenol; 6. ferric oxalate; 7. hydrogen.

plant or in the bacteria oxidizes some hydrogen donor (H_2S) supplied externally. According to this view photosynthesis was represented by the following equations:—

$$
\begin{aligned}
& 4H_2O + 2A + 4X \rightarrow 2AH_2 + 4(XOH) \\
& CO_2 + 2AH_2 \rightarrow (CH_2O) + H_2O + 2A \\
& 4(XOH) + 2H_2O \rightarrow 4H_2O + 4X + O_2 \\
\textit{or}\ & 4(XOH) + 2H_2S \rightarrow 4H_2O + 4X + 2S
\end{aligned}
$$

Thus the chloroplast reaction would then be represented essentially as

$$2A+4H_2O \rightarrow 2AH_2+O_2+2H_2O$$

the reaction only occurring in light and in the presence of a suitable hydrogen-accepting reagent which would react with the reduced compound AH_2.

In order that the hydrogen donor produced can reduce carbon dioxide at the concentration in which it is present in air the oxidation reduction potential of the hydrogen donor AH_2 must be even more negative than the standard hydrogen electrode. It is therefore important to consider the reducing properties of the chloroplast system, conveniently summarized in Table 7.1 and in Fig. 7.2, which shows the relation between the electrode potentials and the percentage reduction of the effective reagents. The reduction of ferric oxalate represents the greatest reducing potential that has yet been obtained in the chloroplast reaction, and in this case complete reduction would only be approached at an oxygen pressure far below atmospheric. This would suggest that at pH 7 and with the highest light intensities yet used the chloroplast reaction has a limiting potential of about zero. If reagents of more negative potential are used with the same high light intensity they will be reduced in appreciable amount only at infinitesimally small oxygen pressures.

Hence chloroplast preparations do not 'split water' to the extent required by the simple formulation of photosynthesis proposed by van Niel. This may be due to the present methods of preparing chloroplast preparations. Alternatively two other interpretations are possible, namely that either (1) the chloroplast reaction *in vitro* is an artefact and has no direct relation to processes of reduction occurring in the living cell, at least with respect to the hydrogen acceptors so far investigated; or (2) the reduction of carbon dioxide requires more than the four equivalents of hydrogen chemically necessary (see Chapter 8). The second interpretation would be analogous with other biochemical systems but would necessitate some modification of the mechanism suggested by van Niel.

Summarizing, we see that illuminated chloroplasts catalyse the production of oxygen from water in the presence of certain hydrogen acceptors in a reaction which shows many of the characteristics of the oxygen-producing reaction in photosynthesis. So far, however, the reducing properties of illuminated chloroplasts are not of sufficient potential to account for a direct reduction of carbon dioxide by reaction with the minimum number of equivalents of hydrogen.

COUPLING OF THE CHLOROPLAST REACTION WITH CARBON DIOXIDE FIXATION

In the preceding chapter, mechanisms of carbon dioxide fixation using reduced TPN were discussed and it was seen that fixation would only occur at moderate concentrations of carbon dioxide if the ratio of reduced to oxidized coenzyme was maintained very high. Attempts to determine whether DPN or TPN was reduced by illuminated chloroplasts were at first unsuccessful; this is in agreement with the view that the maximum observed potential of the chloroplast system is about zero whilst that of the coenzyme is $-0{\cdot}3$ volt. If however the concentration of oxygen is maintained very low then the reduction of TPN can be demonstrated using a carboxylating reaction system such as pyruvic acid, carbon dioxide, manganese salts, and 'malic enzyme'. In this reaction system malic acid is formed when the chloroplasts are illuminated, since TPN is reduced to at least a limited extent and the reduced TPN is removed sufficiently rapidly by the reaction between pyruvic acid and carbon dioxide. Vishniac and Ochoa (1951) demonstrated this coupled reaction between illuminated chloroplasts and 'malic' enzyme obtained from pigeon liver. With $7{\cdot}5\ 10^{-3}$M. pyruvate, $2{\cdot}25\ 10^{-5}$M. TPN and with 10^{-2}M. bicarbonate in the presence of 5% carbon dioxide (pH 7·14) malic acid was produced in a concentration of 3·5 10^{-5}M. after 120 minutes illumination. Other workers (Tolmach, 1951; Arnon, 1951) used 'malic' enzyme from wheat germ and from sugar beet and sunflower leaves. With unwashed chloroplast preparations addition of TPN resulted in a considerable oxygen evolution far in excess of the amount of TPN added. This indicates a coupling of the reduced TPN with other reactants present in the unwashed suspension which have not yet been identified.

We have seen in the previous chapter that if the carbon dioxide were first changed to a carboxyl phosphate, TPN could then effect its reduction to carbohydrate. We have already mentioned that if these 'high energy' phosphate compounds were to be directly produced from light energy the process would be likely to be one of low efficiency. It is therefore more probable that they would arise from the oxidation of some intermediate, possibly the reduced product of the light reaction. The uptake of inorganic phosphate in the light for both *Chlorella* and the photosynthetic bacterium *Chromatium* is not very different from that in the dark. There is some evidence for a change in distribution between different phosphate compounds with an increase in ester phosphate which is less in the presence of carbon dioxide or of glucose. More recently Strehler (1953) has demonstrated marked changes in ATP in the green cell upon illumination. The significance of such changes will arise in the discussion of the mechanism of photosynthesis.

CHAPTER 8

THE MECHANISM OF PHOTOSYNTHESIS

(A) THE LIGHT REACTION

Conversion of light into chemical energy

A DESCRIPTION of the mechanism of the conversion of light energy into a chemical form by the green plant presents a problem which lies on the borderline of physics and chemistry. This may be true of all the cases of energy conversion from one form to another when they are associated with living organisms. The most complete understanding of such processes can best be reached in terms of the experimentally observed properties of the isolated components in relation to one another, provided that these relationships can be proved to hold in the living cell.

The brilliant researches of Willstätter, the insight of Küster, and the chemical studies of H. Fischer demonstrated how the pigment chlorophyll may be isolated, its molecular structure determined and its properties studied. The wealth of chemical and of physical information now available cannot however wholly be applied to the pigment as it exists in the cell. This is because once the pigment has been isolated in pure form it has not been found possible to have it in the same state as it is in the living cell. As we discussed in Chapter 2 chlorophyll is present, apparently, in combination with some specific cellular constituent, very possibly a protein. This complex has not yet been isolated in an unmodified form from the plastids, in contrast with the haem protein compound haemoglobin which has been isolated from the red blood corpuscle and for which it has been possible to relate the properties of the pure substance to its physiological function. It follows that the properties of chlorophyll alone can give but a partial indication of the mechanism of its function in the cell. Nevertheless the development of knowledge concerning all the light absorbing pigments is an essential step in the study of photosynthesis.

When light is absorbed by the pigment system in a plant, theory would suggest a number of alternative mechanisms by which the energy of the quantum of light is transformed. In general, with light absorbing substances, the energy is degraded into heat. With a 'black' substance this may be virtually complete for radiation of all wavelengths as with the thermopile surface (used for measurements of radiant energy). There is however little opportunity for observing what is going on in the absorption of light by a black body. Fortunately, however, many of the pigments associated with photosynthetic mechanisms show, both in the living cell and in organic solution, the property of fluorescence.

Fluorescence of pigments

The fluorescence of chlorophyll shows a spectrum which is independent of the wavelength of the light absorbed. This means that there is no characteristic fluorescence associated with the higher energy transition which would result from the absorption of blue or violet light. The necessary condition that the wavelength of the exciting light must be shorter than the wavelength of the emitted light is shown by the maximum of the fluorescent spectrum being of a longer wavelength than the maximum of the absorption band of chlorophyll in the red. With chlorophyll *a*, for example, we may write

$$\mathrm{Chl}+h\nu_1 \rightarrow \mathrm{Chl}^*;\ \mathrm{Chl}^* \rightarrow \mathrm{Chl}+h\nu_2$$

where ν_1 is the frequency absorbed and ν_2 the frequency corresponding to the fluorescence spectrum and Chl* represents the 'fluorescent state' of the molecule which reverts to the normal state with the emission of light.

The energy corresponding to the fluorescent state remains within the pigment molecule for a time which is long compared with the frequency of the exciting light; the half life of the fluorescent state may be measured in the following way. If the pigment is dissolved in a viscous medium and exposed to polarized exciting light then the fluorescence is found to be polarized. This is because the excited molecules have not appreciably altered their orientations by thermal molecular movement before the light emission. As the viscosity is lowered so the polarization of the fluorescence is diminished. If the molecular dimensions of the pigment are known then the degree of disorientation with time may be estimated, and thus the time of half life of the fluorescent state be deduced. For many substances the half life is of the order of 10^{-8} sec. (compared with the reciprocal frequency of light 10^{-14} sec.).

With solutions of chlorophyll only a fraction of the absorbed light is emitted as fluorescence (about 10%), while in the living plant the fluorescence yield is still smaller, being less than 1%. From the point of view of conversion of light into chemical energy the fluorescence represents a total loss. But the investigation of the fluorescent radiation is of great value for it gives a means of measuring the relative amount of Chl*, provided it can be shown or inferred that the fluorescent yield remains unchanged.

Energy transfer between pigment molecules (*in solution*)

With many substances the fluorescent yield is reduced by increasing concentration, even after allowing for self-absorption of the fluorescence resulting from overlap with the absorption spectrum. In addition it has been found that in a viscous solvent the degree of polarization remaining in the fluorescent light also is lessened by increase in concentration. But the important point is that the degree of polarization begins to fall off before the total fluorescence yield is affected. The interpretation of this result is that the energy of the fluorescent state can be transferred as a whole from one pigment molecule to another, so that molecules in different orientation contribute to the emitted light resulting in a decrease in the degree of polarization. The mechanism of the energy transfer between the molecules is, according to Th. Förster (1949), by the process of inductive resonance. It has been shown that energy may be transferred between molecules separated by a distance of the order of 60 Å.

Now when two pigments are present in a solution it is sometimes found that the light absorbed by one pigment will excite the fluorescence of the other. Chlorophyll *a* and *b* show this phenomenon of sensitized fluorescence. In a mixed solution the fluorescence is nearly all that of chlorophyll *a*. This has been studied in relation to concentration by Duysens (1952). The results were in agreement with the calculations of Förster and showed that with concentrations of pigment approaching those in the chloroplast the efficiency of energy transfer from chlorophyll *a* to chlorophyll *b* could indeed be 100%. In *Chlorella* although chlorophyll *b* is present it does not show fluorescence in the cell and Duysens concludes that the transfer of energy from chlorophyll *b* is nearly 100% within the cell. We may represent this transfer as

$$\text{Chl } b^{*} + \text{Chl } a \rightarrow \text{Chl } b + \text{Chl } a^{*}$$

The transfer of energy by inductive resonance depends on there being an overlap in the wavelengths of the fluorescence spectrum of the first pigment (Chl *b*) with the absorption spectrum of the second pigment (Chl *a*). While this overlap is essential for inductive energy transfer, the actual presence of an overlap does not tell us whether a given pair of pigments will show sensitized fluorescence. When there is no observed overlap in the spectra it has been shown in certain cases that energy may be transferred provided the molecules are in contact, as in a molecular chemical compound. (For example, the haemprotein CO compounds are dissociated by light not only by wavelengths absorbed by the pigment, but also by wavelengths absorbed by the protein.) The distinguishing feature of the inductive transfer is that it can operate over an intermolecular distance.

Energy transfer in the chloroplasts

The quantum efficiency of photosynthesis was determined by Emerson and Lewis for *Chlorella* and shown to be nearly constant over a range of the spectrum which includes the absorption bands of both chlorophyll *a* and *b*. This must imply that both the chlorophylls are equally effective in photosynthesis. Further as we have seen in Chapter 4 in the brown algae, the blue-green algae and the red algae the light absorbed by pigments other than chlorophyll is also efficiently used in photosynthesis. Thus light absorbed by the carotenoid fucoxanthol was found to be as efficient in photosynthesis as the light absorbed by chlorophyll *a*; also in the living cell the absorbed light is equally efficient in sensitizing the fluorescence of the chlorophyll. In the blue-green and the red algae similar relationships are found between phycoerythrin and phycocyanin and chlorophyll *a*, but in these algae experimental data have not been obtained to show that energy transfer occurs only by inductive resonance.

In summary, it has been found experimentally that there is a transfer of energy in a physical sense between the pigment molecules in the chloroplast. This fact leads to the important question: with what substance, a pigment or otherwise, does the 'physics' end and the 'chemistry' begin.

The photochemical cycle and the first chemical step

Chlorophyll *a* is the one photosynthetic pigment which is common to all plants including the various classes of Algae. The possibility therefore exists that chlorophyll *a* or some fraction of it can be regarded as the essential photochemical catalyst. Moreover French and Young (1953) have pointed out that it is chlorophyll alone which shows characteristic induction effects in intensity of fluorescence (with constant illumination) in the living cell; in the red algae for example the phycoerythrin and phycocyanin show no fluctuations in the intensity of their fluorescence. This appears to place chlorophyll *a* in a unique position with regard to the other pigments. Thus it may be said that with chlorophyll *a* we transform light energy from a physical point of view. Perhaps, then, this one pigment may pass through a cycle of chemical change.

Physico-chemical theories of the transformation of light energy

The function of theory developed on these lines is to direct and guide experiments concerned with the discovery of how the absorption of light energy and its conversion into a chemical form is to be explained in terms of the details of molecular structure in the photocatalytic system. Here the principles are largely derived from the successful application of quantum theory to atoms and to very simple molecules. More recently however the theory has been applied to certain types of organic substances with considerable success.

The discovery of the 'triplet state' in the photochemistry of fluorescein by G. N. Lewis and Kasha has had an important bearing on theory in this branch of photosynthesis. The triplet state of a molecule differs from the fluorescent state in giving a more delayed light emission, known as phosphorescence. The process is not influenced by temperature in the same way as is fluorescence. Chemically a molecule in the triplet state should have the properties of a biradical and thus exhibit a definite paramagnetic property. This was proved in the case of fluorescein and further the triplet state could be recognized by a characteristic absorption spectrum. In passing from the fluorescent state of the molecule to a triplet state some energy has to be lost, and with fluorescein the energy level is appreciably below that of the fluorescent state. So far evidence for the existence of triplet states has been obtained with only a limited number of organic compounds. Hence whilst the existence of the triplet state would seem to offer the key to determining the mechanism of conversion of light into chemical energy we have as yet no conclusive evidence that such a state is possible in the case of chlorophyll *a*. Even so the conception of a 'biradical' formed from a molecule consequent on light absorption is helpful in considering the impact of physical theory upon our more specialized biochemical field. Such a biradical would have both oxidizing and reducing properties. Thus perhaps it could easily lose the extra energy by chemical reaction with both an electron donor and an electron acceptor. Also the molecule might react with itself to give two products the reduced and the oxidized form of the dye. Hence we may see how a general postulate has come to be accepted by many: that the action of light is to produce say, with some compound XY, or with a complex of two compounds, X and Y the products XH and YOH, both being regarded as radicals, analogous to semiquinone compounds. We cannot yet identify either X or Y in the case of chlorophyll; nor has it been shown that X represents CO_2 in some stage of reduction. Yet we can see how this general scheme expresses the light process as electron transfer and is compatible with the idea of hydrogen transfer in photosynthesis. Further there is no reason why H and OH could not arise from water, a formulation consistent with the general theory developed by van Niel (Chapter 5).

Such a formulation has been successfully applied by Gaffron (1944) to the interpretation of his experiments with Scenedesmus. In photoreduction the XH and YOH result from the action of light; in the oxyhydrogen reaction the YOH is derived in dark from the oxygen itself.

Studies with the photochemistry of chlorophyll solutions have not only shown the existence of photochemically induced reversible states of the molecule but also under appropriate conditions the formation of a reversibly oxidized form of chlorophyll. Recently the existence of a reversibly reduced form of chlorophyll (in pyridine as a solvent) has been demonstrated. A reversible bleaching of bacteriochlorophyll *in vivo* has also been demonstrated. However it has not been established whether chlorophyll actually does undergo a reversible chemical transformation which itself is an essential part of photosynthesis.

Energy loss in back reaction

In the consideration of any possible detailed molecular mechanism of the photochemical reaction it is necessary to postulate some efficient means of preventing an immediate dark reaction the reverse of that initiated by light. This is because the process of photosynthesis is found to use the light energy with high efficiency at low light intensities. The chloroplasts and indeed the individual grana themselves have been shown to have a characteristic organized structure. It may well be that this structure together with specific enzyme systems, which doubtless form part of it, will eventually provide an explanation as to how the great tendency for the reversal of the light-initiated reaction is restrained. The discovery of a back reaction both in the living cell and in chloroplast preparations which leads to light emission was recently made by Strehler and Arnold (Chapter 4). This seems to complete the biological picture of photosynthesis in a remarkable way: so many reactions in the living cell are reversible and this may now have been shown to be true, in one sense, of the absorption of the light itself.

(B) HYDROGEN TRANSFER IN PHOTOSYNTHESIS

Historical

Leaving now the consideration of the light absorbing systems of the plant and taking the fact of the conversion of light into chemical energy for granted, the problem of photosynthesis may be considered in terms of the purely chemical transformations which lead to the reduction of carbon dioxide to carbohydrate. This approach can develop only with the gradual discovery of all the components and the definition of the course of the chemical reactions. Ever since the development of biochemistry as a discipline of its own the justification for the old theories based on the formation of formaldehyde began to look inadequate whilst the purely kinetic theories became unsatisfying. Biochemical fields had been widely explored and thus even though they were faced by the lack of any active isolated systems from the green plant, Kluyver and van Niel had by the early 1930's been able to put the problem of photosynthesis in a biochemical perspective (see Chapter 5). A few years later when it was found that light could be converted into energy in a chemical form by cell-free extracts from the green plant, i.e. the chloroplast reaction, the direct biochemical approach to photosynthesis was made possible.

The in vitro *production of oxygen*

The chloroplast system produces oxygen under the influence of light so long as it is supplied with a suitable hydrogen acceptor. The maximum free energy change that has been found possible under conditions where oxygen is being produced corresponds to a reducing potential of about zero at pH 7, referred to the normal hydrogen electrode, i.e. to a value of +12 on the rH scale and to 76,000 cals. per O_2 molecule. $2A+2H_2O \rightarrow O_2+2AH_2$. The mean energy for the quantum of light corresponding to the fluorescence of chlorophyll *a* in the plant is about 41,000/*N* cals. (N = Avogadro number). In the over-all reaction,

$$CO_2+H_2O \rightarrow (CHOH)+O_2$$

the *mean* free energy change *per H* transferred (4H are involved for each (CHOH) produced) is about 28,800 cals., i.e. 115,000 cals. per O_2 molecule. This corresponds to a reduction potential at pH 7 of −0·5 volt or to a value of about 5 units on the rH scale. Hence no direct reduction of carbon dioxide can result in the chloroplast reaction. Thus as Rabinowitch has stated, the chloroplast reaction can only represent one half of photosynthesis.

Physically the active system represents from some plants, minute fragments of the chloroplasts, whereas in others whole chloroplasts alone preserve activity. The particles derived from the fragmented chloroplasts may be obtained many times smaller than the individual grana of the chloroplasts and still show a measurable activity. Even at this level the particles are very complex chemically. They contain all the known pigment components of the chloroplasts, the chlorophylls, the carotenoids and the characteristic cytochrome components. Even if the chloroplast preparations are amply supplied with a hydrogen acceptor, the oxygen production still shows the phenomenon of light saturation, thus indicating that at least one dark process is concerned in addition to the light reaction.

If this subcellular system is accepted as being a part of the intracellular mechanism *in vivo* then the property of evolution of oxygen together with the simultaneous transfer of hydrogen possessed by it may be considered to be part of the mechanism of photosynthesis. If so, it would appear that CO_2 is not directly concerned in oxygen production as studies of the direct response of the illuminated plant had at one time suggested. Thus the experimental possibility of producing oxygen by means of the chloroplast preparations provided support for the generalization of the process of photosynthesis first announced by van Niel. It follows from this theory that in the green plant water itself is the ultimate hydrogen donor, and evidence for this using isotopes has been discussed (see Chapter 7).

Comparison of the chloroplast reaction with an oxidase in respiration

In respiration oxygen is the ultimate hydrogen acceptor and the water originally used in photosynthesis is formed again. In actively respiring cells of both plants and animals oxygen is reduced through the cytochrome system. This system, discovered by D. Keilin, may be prepared as a very fine particulate suspension and is derived from mitochondria. The component cytochrome *c* does not react directly with oxygen but is immediately oxidized if added to a mitochondrial preparation containing cytochromes *a* and a_3. If succinate is added in absence of oxygen the cytochrome *c* is immediately reduced. The hydrogen transport from succinate to oxygen occurs in steps. The complete step from succinate-fumarate to cytochrome *c* would correspond to 0·27 volt; the step from cytochrome *c* to oxygen 0·53 volt.

The active chlorophyll-containing system derived from the chloroplasts when illuminated will reverse the action of cytochrome oxidase; water is oxidized to oxygen. The 'hydrogen' has then to reduce a hydrogen acceptor. A simple experiment serves to illustrate the complementary nature of the two systems. Cytochrome *c* is added to a crude chloroplast preparation from, say, the pea plant. Chloroplasts and mitochondria are both present. The oxidation in the dark and the reduction in the light of the added cytochrome *c* is easily followed by the observation of the visible absorption spectrum. The sharp absorption band of reduced cytochrome *c* (550 mμ) appears during an interval of illumination and temporarily disappears during a dark interval.

Chloroplasts and mitochondria have several points in common and biochemical evidence suggests that the process of photosynthesis may quite closely resemble a reversal of respiration.

The reversibility of enzyme systems concerned in respiration

In heterotrophic organisms respiration can in a sense reverse itself; carbon dioxide can be re-assimilated and appear even in carbohydrate. This has been shown for mammalian liver both in tissue slices and in the intact animal. Naturally, of course, there can be no net assimilation of carbon because the reincorporation of the CO_2 requires energy derived from respiration itself. In the green plant however there is a supply of energy from the photochemical transformation.

The process of respiration considered as a whole is to be regarded as an irreversible reaction because the over-all change in free energy is large. If this is represented by the complete oxidation of carbohydrate the value is about 115,000 cals. per CHOH group. Thus the amount of carbohydrate theoretically in equilibrium with CO_2 and O_2 at atmospheric pressure would be $10^{-84\cdot 4}$ molar, an amount equal to zero for all practical purposes. The respiration of carbohydrate takes place in many stages and each stage will correspond to a smaller energy step. When in a given step the concentrations of both an initial and a final product remain large enough to be within the range of an enzyme system concerned in the catalysis of the reaction the process can become reversible from a practical standpoint. For example, in the presence of inorganic phosphate starch may break down so completely that no residual starch may be detected. This process consists of two steps each associated with a small free energy change. For each step, in presence of the appropriate enzyme, equilibrium may be approached from either side.

$$\text{Starch}+\text{phosphate} \rightharpoonup \text{glucose-1-phosphate} \qquad (1)$$
$$\text{Glucose-1-phosphate} \rightharpoonup \text{hexose-6-phosphate} \qquad (2)$$

If now, starting with hexose-6-phosphate, some means of almost complete removal of inorganic phosphate is supplied, both steps may be reversed and starch is formed again. This was accomplished *in vitro* by Cori by having barium ions present, the barium phosphate being sufficiently insoluble. The increase in free energy with reference to the carbohydrate is at the expense of an irreversible reaction, the precipitation of barium phosphate. In the living cell there is a very efficient way of removing inorganic phosphate, namely, oxidative phosphorylation. The significance of this in the present case depends in part on respiration being regarded as an irreversible change with reference to the starch hexose phosphate system. Therefore in order to reverse any reaction the energy supplied for removing a product is at the expense of an irreversible reaction in another system starting far from its position of equilibrium. This then gives a mechanism of direct reversal of an enzyme catalysed reaction but only relatively small energy steps may be reversed in this way. Another example of a reverse reaction is the formation of starch from glucose. Starch is broken down to glucose by alpha- and beta-amylase together with maltase. Here the action is hydrolytic and it would be difficult to conceive of a direct reversal in dilute aqueous solution. The reaction may be reversed in water by an indirect mechanism. This possibility depends on the resistance of organic phosphate compounds to spontaneous hydrolysis and on the direct transfer of the phosphate group. The glucose in presence of hexokinase and adenenosinetriphosphate (ATP) is converted into hexose phosphate.

$$\text{ATP}+\text{glucose} \rightharpoonup \text{glucose-6-phosphate}+\text{ADP} \qquad (3)$$

Then starch may be formed by the reversal of the reactions (2) and (1) as previously described. The process of oxidative phosphorylation both removes the phosphate and regenerates the ATP and the energy is derived from respiration.

As a third example of reversal of a process, the reduction of phosphoglyceric acid to triose phosphate introduces another principle. The potential of the system triose phosphate/phosphoglyceric acid at pH 7 would be about −0·48 volt. When phosphoglyceric acid reacts with ATP in presence of the appropriate phosphokinase diphosphoglyceric acid is formed, from which triose phosphate may easily be formed with triose phosphate dehydrogenase in presence of reduced DPN (reducing potential of about 0·32 volt) whilst inorganic phosphate is liberated. The triose phosphate system is described in Chapter 6 and it is clear that the energy involved in bringing the phosphoglyceric acid into equilibrium with the coenzyme is derived from the phosphate group. The free energy of hydrolysis of ATP to ADP is taken as −11,300 cals. under standard conditions. If no energy is lost by the spontaneous hydrolysis of the diphosphoglyceric acid there could be a potential shift of 0·2 volt when the reduction of phosphoglyceric acid is compared with the reduction of diphosphoglyceric acid.

Thus given the appropriate catalytic mechanisms there is the possibility that any of the steps in respiration may be reversed, but such a reversal must always be accompanied by a corresponding gain in free energy, the energy being derived from the over-all respiration.

Reduction of coenzymes by light

The experiments with chloroplasts suggest that energy partially equivalent to that derivable from respiration can be obtained by transformation of light energy into a chemical form; the available energy may be represented as a redox potential. This discussion of reversibility of reactions in connexion with respiration has been given to indicate the far-reaching importance of Ochoa's suggestion, later confirmed by his experiments that a possible representation of the process of assimilation of CO_2 could be the reduction of coenzymes by the illuminated chlorophyll containing system of the chloroplasts. Experiments showed that no perceptible direct reduction of TPN (as determined spectrophotometrically) would occur simply in presence of illuminated chloroplast preparations. This was to be expected from the data on the production of oxygen, though of course there is no justification for accepting the present experimental measurements on the reducing properties of the chloroplasts as final: the present measurements are, however, consistent with a reduction of the coenzymes to only a very small extent.

The discovery that the chloroplast reaction may be coupled with known respiratory systems through the coenzymes greatly extends the biochemical methods available for the study of photosynthesis. In the original model experiment of Vishniac and Ochoa (mentioned earlier in Chapter 7) the complete malic enzyme system obtained from pigeon liver together with pyruvic acid was added to an illuminated preparation of spinach chloroplasts. The conditions were maintained anaerobic by removal of the oxygen by chromous chloride in a separate compartment in the reaction vessel. The oxygen produced was then measured in terms of the chromic chloride and found to correspond with the amount of malate formed from pyruvate. Carbon dioxide was supplied as bicarbonate with 5% CO_2 in the gas phase. In this experiment the essential reaction brought about by the illuminated chloroplast preparation must be:

$$\mathrm{TPN^+ + H_2O \rightarrow TPNH + H^+ + \tfrac{1}{2}O_2};\ \Delta F = 50{,}800 \text{ cals.}$$

Ochoa found that other systems dependent on coenzymes could be reversed. For example, pyruvate could be reduced to lactate, by the dehydrogenase depending on DPN, in presence of the illuminated chloroplast preparation. It was also found that the mitochondria from the mung bean would carry out oxidative phosphorylation when in presence of DPN and the illuminated chloroplast preparation. It is thus established that coenzymes I and II can be reduced by chloroplast preparations when a suitable system is also present to accept the 'H' originally resulting from the photochemical reactions. This allows measurements to be made of the extent to which a reversal of respiratory process can occur with chloroplasts *in vitro*. When the experiments with chloroplast preparations are carried out under anaerobic conditions and all the oxygen is removed the results obtained can be explained in terms of the measurement of the reducing properties of the chloroplasts by other methods.

But this would necessitate the oxygen being removed efficiently down to the level of 10^{-8} mm. Hg pressure of O_2; that is, if the chemical energy available from the chloroplast reaction is taken as for the reaction:—

$$R+H_2O=RH_2+\frac{1}{2}O_2+38{,}000 \text{ cals.}$$

Arnon (1951), in his experiment with the formation of malate from pyruvate, used a malic enzyme preparation from leaves and was able to measure the oxygen produced manometrically. The amount of malic produced was in about the same proportion to the pyruvate as in the experiments of Vishniac and Ochoa. The final pressure of O_2 would have been 0·75 mm. Hg pressure. This would require the chemical energy from the chloroplast reaction to be 41,000 cals. and represents a discrepancy of 3,000 cals. on the other methods of measuring the chloroplast reaction. We could dismiss the other measurements as unreliable, but even so the question of how much energy is directly made available in the chloroplast reaction would be far from solved. The green plant (whilst maintaining a pressure of O_2 above even that in the atmosphere) can carry out reductions which involve a much greater total amount of energy than is represented by the formation of malate from pyruvate to the extent observed *in vitro*.

Thus the present position is that the chloroplast reaction *in vitro* may correspond to the oxygen-producing mechanism of photosynthesis while it does not correspond directly to the reducing mechanism concerned in the assimilation of carbon dioxide. This latter statement would imply either that the substances of the type R cannot *in vitro* become appreciably reduced because of the back reaction

$$RH_2+O_2=R+H_2O_2$$

or that the substances of the type RH_2 are not strong enough reducing agents, as their standard redox potentials are too positive. Both these considerations limit the conclusions about the mechanism of photosynthesis that may be drawn at present from *in vitro* experiments.

(C) REDUCTION OF CARBON DIOXIDE

Theory of van Niel

The simplest assumption that could be made concerning the mechanism of photosynthesis on the basis of the general scheme of van Niel would be that the assimilation of CO_2 to give carbohydrate requires only the four equivalents of H resulting from the production of one molecule of oxygen per (CHOH) group. This assumption has strong support from the fact that the photosynthetic quotient approaches unity under a variety of conditions. Further support might be derived from the fact that the tangent to the origin of the light curves of photosynthesis and of the chloroplast reaction in terms of oxygen production appear to have the same value, indicating that the amount of light energy required for the production of one oxygen molecule from water is the same whatever the amount of chemical energy fixed in the form of a reduced compound. If more than the four equivalents of H were required to reduce one molecule of CO_2 we might hope to find a system which would produce oxygen with less light energy than is required for photosynthesis.

A chemosynthetic mechanism in addition to the photochemical process

In spite of the fact that this simple assumption appears to have good experimental support, the evidence derived from comparative studies with different organisms would suggest that it would be more correct to assume that more than the four equivalents of H in the form of a reducing agent are involved for each molecule of carbon dioxide. This would lead to the hypothesis that in the green plant the photochemical processes are completed by a dark process equivalent to chemosynthesis. If molecular oxygen were concerned in this the reaction with CO_2 might be of the following type:—

$$n(\mathrm{R})+n(\mathrm{H_2O})+\text{light}=n(\mathrm{RH_2})+(\tfrac{1}{2}n)\mathrm{O_2} \qquad (\alpha)$$

followed by a dark process

$$n(RH_2)+CO_2+\left(\frac{n}{2}-1\right)O_2=(CHOH)+n(R)+(n-1)H_2O \qquad (\beta)$$

where the reduction of CO_2 is coupled with the reoxidation of RH_2. The equation α and β will represent the absolute minimum when $n = 2$ (i.e. transfer of 4 equivalents of H). If it were possible to define the number n in the equations as represented, i.e. to decide if the third term in (β) were either absent, molecular oxygen, or some equivalent oxidant produced in a light reaction, the analysis of the mechanism of photosynthesis would be much advanced. There is no convincing direct evidence at the moment to suggest that n is necessarily greater than 2. On the other hand such a 'chemosynthetic hypothesis' gives the most plausible method of relating the present results of *in vitro* experiments with the physiological process of photosynthesis. If, for example, the view of Ochoa is accepted that the essential result of the photochemical process is the reduction of the coenzymes (representing R in the above equations) then, in order to reduce the CO_2 from the atmosphere to the state of carbohydrate in the plant, a further supply of chemical energy is needed. This further supply could of course only come from light and thus some form of chemosynthetic coupling must be postulated. This is because the reoxidation of the coenzymes is only appreciable around the range −0·3 volt at pH 7. The reduction of CO_2 would require it to be in the range −0·5 volt. The definition of this amount of extra energy makes Ochoa's experiments all the more suggestive, for the gap to be bridged is within the range of free energy increase by means of oxidative phosphorylations, represented by a potential of 0·2 volt, a possibility suggested by Ruben and many others (see Rabinowitch, vol. I).

There is some experimental evidence for the supposition that the reduction of coenzymes caused by the illuminated chloroplast preparations is by no means a direct process. It is also possible that it requires some extra energy. Such an assumption would, for example, reconcile the experiment of Arnon with the reducing properties of the chloroplast observed by other methods. It would be in accord with the experiment of Tolmach (mentioned previously on p. 125), who showed that at very low O_2 pressures the addition of TPN to illuminated chloroplast preparation produced much more O_2 than would correspond to the reduction of the co-enzyme. This, however, might lead back to the extreme view that the energy fixed by light is only equivalent to a reaction of free energy approximately equal to the oxidation of succinate to fumarate by O_2. ($E_0' = 0{\cdot}0$ volt.) The oxidation of succinate by mitochondria can lead to phosphorylation with a quite high efficiency. An elucidation of the mechanism of the process would be of interest in the present connexion; though there is no evidence to show that the chloroplasts react directly with succinate or fumarate.

Experiments of Warburg and Burk

Some of the results obtained from physiological measurements on living cells have been quoted in support of the chemosynthetic hypothesis. Warburg's discovery that oxygen is required for the uptake of CO_2 by illuminated *Chlorella* cells is most easily interpreted in this way. The influence of cyanide as an inhibitor of the uptake of CO_2 in light can be interpreted as due to the blocking of an oxidation reaction. In this connexion the discovery of the adaptation of the alga *Scenedesmus* to hydrogen made by Gaffron is relevant. This organism can be adapted to oxidize hydrogen by molecular oxygen and assimilate CO_2 in the dark. Gaffron measured the n referring to the equation β (p. 142) and found a value of 6. In the absence of CO_2 it was found that the oxidation of H_2 was slower and there was evidence that peroxide accumulated. (Compare the Mehler reaction with chloroplast preparations (Chapter 7).) In the absence of oxygen and at low light intensities the H_2 is oxidized to water during the photoreduction of the CO_2. If the oxygen-producing mechanism of the cells is inhibited (for example by *o*-phenanthroline) photoreduction may proceed in strong light; but normally if the light intensity is increased photoreduction is at once replaced by photosynthesis with the evolution of O_2. If now equation β holds with $n = 2$ then it must be supposed that in the photoreduction by *Scenedesmus* oxygen is replaced by another oxidant resulting from the light reaction, and that the same situation may also apply to photosynthesis.

Several years ago G. E. Briggs pointed out that if some chemosynthetic process was taking place in photosynthesis the gas exchange would not be linear at the beginning of either light or dark periods. There should, for example, be an increase of O_2 production and a deficiency of CO_2 uptake at the beginning of a light interval; it being assumed that the induction effects were sufficiently small. Quite recently as mentioned in Chapter 4, Burk and Warburg (1951) have some evidence for such an effect. Their experiments with *Chlorella* were carried out with alternate light and dark (or compensating) intervals of the order of 1 minute. It was concluded that there was a greatly increased output of oxygen in proportion to the absorbed light energy at the beginning of the light interval. However, in contrast to the possibility envisaged by Briggs, there was an equal increase of uptake of CO_2. Then during the dark interval there was initially a greatly increased O_2 uptake together with a corresponding increase in CO_2 production over and above that of the dark respiration measured over a long period. The net photosynthesis over the light and dark period taken together was the same as that observed under continuous illumination.

The conclusions of Burk and Warburg are based on minute changes in the level of the manometric fluid rapidly read with vigorous shaking throughout the short alternate intervals of illumination. It is difficult to see how the gas liquid equilibrium could be established to allow results of the accuracy claimed to be obtained during short intervals. Against this criticism it must be emphasized that it is possible when using a well-tried method to make qualitative deductions beyond those apparent from the formal statement of the actual measurements: many discoveries have been initiated in this way. Confirmation of these results by other methods is awaited.

It seems probable that under most conditions the photosynthetic system would be so well integrated that the opposite phases of activity could not be separated, which would account for these transitory gas exchange effects not being apparent in previous experiments with *Chlorella* or other plants.

Further, Brown (1953) and colleagues have found that with *Chlorella* cells the consumption of oxygen from the gas phase is not generally affected by light and dark, i.e. the total uptake of O_2 from the gas phase is the same in light and dark. This would indicate—if $n > 2$ in equations α and β—that the extra oxygen does not appear as such. In other words, $2n$ will represent the number of electron transfers involved per equivalent of CO_2 converted to the level of carbohydrate.

Cytochrome components in chloroplasts

The chloroplasts have been shown to possess cytochrome components (Chapter 3). The component cytochrome *f* is different from cytochrome *c* in its physical properties; the haematin group and the properties of oxidation and reduction are similar in the two pigments. The chloroplasts also contain a haem protein which is similar to component cytochrome *b*. In respiration there is a stepwise transfer of H or of electrons to the level of molecular oxygen. Davenport and Hill have suggested that in the chloroplast there could also be a stepwise transfer of H or electrons from the level of oxygen to that of a reducing agent. They found that the redox potential of cytochrome *f* was mid-way between the potential of molecular oxygen and the measured reducing potential of the illuminated chloroplast. Thus the chloroplast reaction might consist of two steps, each step being dependent on a light reaction. The oxygen molecule would be supposed to arise by the reduction of cytochrome *f* which itself becomes oxidized when the substance characteristic of the second step becomes reduced. It happens that the energy for the reaction (pH 7·0)

$$4 \text{ cytochrome } f\ (Fe^{3+}) + 2H_2O = 4 \text{ cytochrome } f\ (Fe^{2+}) + 4H^+ + O_2$$

corresponds almost exactly with the mean energy of the quanta of light of the fluorescence spectrum of chlorophyll *a* in the plant. If the light energy is used efficiently it might be possible to observe under some condition the production of O_2 with a quantum efficiency of one. This is actually what Warburg has claimed from the results obtained during the alternate light and dark intervals with *Chlorella.* But any theoretical approach here needs to be guided by more experimental results.

Interpretation of quantum efficiency measurements

There does not seem to be any evidence from physico-chemical knowledge that more than one electron could be transferred for each quantum of light against an energy gradient. The transfer of one hydrogen equivalent could provide a very strong reducing agent which could theoretically reverse any of the known enzyme systems concerned with the fixation and reduction of CO_2 to the level of carbohydrate. But the presence of the cytochrome components may indicate that some process involving a stepwise transfer of electrons occurs during photosynthesis. The transfer in this second hypothetical case would be in the direction of a more oxidizing potential as it is in respiration. Only if it is assumed that the characteristic cytochrome components in the chloroplasts are inactive, can the simplest formulation of photosynthesis, as given by van Niel (with $n = 2$ in equations α and β), represent the process in biochemical terms.

This discussion was given in order to show that at the present moment little is known concerning the actual mechanisms operating in the light dependent reactions. If it is finally found that n in the equations α and β turns out to be more than 2, then, from a theoretical standpoint, the term quantum requirement of photosynthesis has little meaning. On the other hand from the physiological point of view the term may be used in a perfectly definite sense. The evidence that the plant in general requires from 8 to 12 quanta of light per CO_2 molecule for the overall process is overwhelming: the question of minimum requirement is still a controversial one. The study of partial reactions which depend on light absorption may resolve the difficulties in the interpretation of experiments with living cells.

(D) PATH OF CARBON IN PHOTOSYNTHESIS

It seems, then, that the study of photosynthesis has opened up a biochemical field of oxidations and reductions comparable with that of respiration. There is, however, a different problem awaiting solution, namely the determination of the sequence of carbon compounds which belong specifically to the process of photosynthesis. The knowledge we now possess is due to a brilliant series of investigations by Calvin and co-workers (1949, 1951) and of Gaffron and colleagues (Fager and Gaffron) discussed in Chapter 6.

One of the difficulties of this approach is that when these methods are applied to the living cell the intermediates of greatest interest are likely to be present in the lowest concentration. Nevertheless the initial findings in Calvin's work of the extreme rapidity with which the carbon of the assimilated CO_2 was incorporated into a large variety of cellular metabolites, gave a new impetus to the study of metabolism in the plant. For example, it was found that in *Scenedesmus* fats and not carbohydrate were formed from acetate in the light. With CO_2 over a period of a few minutes labelled amino acids and corresponding carboxylic acids as well as carbohydrate were formed. In the dark there was a similar incorporation of the CO_2 though at a much slower rate.

With the exposure of preilluminated cells to $^{14}CO_2$ for only a few seconds most of the radioactivity is found in the phosphate ester fraction. Using a method of extrapolation back to zero time it was concluded that phosphoglyceric acid was the first stable derivative of labelled carbon dioxide and that the carboxyl group was labelled first. The same conclusion was reached later by Gaffron in independent experiments. This finding was consistent with the initial labelling of the hexose found in the 3- and 4-positions. Recently Fager (1952) has shown, using the tracer method, that phosphoglyceric acid (PGA) is formed *in vitro* by cell free preparations of spinach leaves. For this a cell extract was prepared in the dark under practically anaerobic conditions. Liquid nitrogen was used to freeze the leaf tissue initially. The extracts contained chloroplast material plus the soluble cell constituents together, no doubt, with particles derived from the mitochondria. The insoluble residue alone was found to be inactive but activity could be restored by addition of the soluble portion. The soluble portion itself could be fractionated by precipitation with acetone and a fraction added to the insoluble which would restore the activity to the same extent as the untreated cell extract. With this *in vitro* system PGA was formed in the dark but the effect could be doubled by exposure to light. The activity of this *in vitro* system when compared with the rate of photosynthesis shown by a plant was extremely small. Nevertheless this discovery of Fager may well lead to a more complete knowledge of the nature of the process of formation of PGA from CO_2. This would be important for the following reason. The rapid labelling of PGA found by Calvin to always occur with normally assimilating cells requires the postulation of something equivalent to a two-carbon compound which has to be supplied by a cycle of chemical transformations. Such a cycle of chemical changes is found in the widely present transketolase and transaldolase mechanisms for the oxidation of carbohydrate through 6-phosphogluconic acid. If these group transfer mechanisms lead to an acceptor for the uptake of CO_2 with phosphoglyceric acid as the product, the only reduction step required to bring one equivalent of CO_2 to the state of carbohydrate would be the reduction of two equivalents of phosphoglyceric acid. With present knowledge of the plant enzymes this reduction step seems possible in the presence of continuously reduced TPN and DPN together with a phosphorylation mechanism. This pathway for carbon assimilation in the green plant could thus depend on the reduction of the coenzymes by the photochemical system as originally suggested by Ochoa. In addition to the single carboxylation step postulated by Calvin, however, it has to be admitted that other possibilities exist for the entry of carbon dioxide into the metabolism of plants.

There are at present good indications of the mechanism of photosynthesis on the basis of the actual carbon compounds concerned. This is the problem of the most general interest for biochemists. The methods of Calvin, when they can be combined with the *in vitro* enzyme studies, can advance our knowledge rapidly.

APPENDIX

VARIOUS UNITS USED IN THE MEASUREMENT OF PHOTOSYNTHESIS

Measurement of light energy

THE *lux* or metre candle is the illumination on a surface normal to the direction of light and 1 metre away from a point source of 1 candle-power.

$$1 \text{ foot-candle} = 10{\cdot}764 \text{ lux}$$

Approximately a tungsten lamp gives an intensity of 2 lux per watt at a distance of 1 metre.

The *total energy flux* is measured using a thermopile or bolometer. It may be compared with that emitted by a 'black body' the total radiation from which at temperature T° Absolute$=5{\cdot}67\ 10^{-5}\ T^4$ ergs/sec./cm.^{2}.

The following units of energy may be used:—

$$1 \text{ gram calorie/sec.} = 4{\cdot}185 \text{ joules/sec.} = 4{\cdot}185 \text{ watts} = 4{\cdot}185\ 10^7 \text{ ergs/sec.}$$

Wavelength of light may be expressed as:—

$$1 \text{ Ångström (Å)} = 10^{-1} \text{m}\mu = 10^{-8} \text{ cm.}$$

The energy associated with one quantum of radiation is given by

$$E = \frac{hc}{\lambda}$$

where h Planck's constant$=6{\cdot}62\ 10^{-27}$ ergs/sec.
c velocity of light$=2{\cdot}99\ 10^{10}$ cm./sec.
λ wavelength in cm.

Approximately a tungsten lamp has total radiation equivalent to 60 ergs/cm.^{2}/sec./lux. About 9% of this energy is at wavelengths less than 7,000 Å so that the visible radiation is approximately 5 ergs/cm.^{2}/sec./lux.

Measurement of photosynthetic activity

The rate of photosynthesis may be expressed in a number of different units, such as mg. gas exchange per dry weight, per fresh weight, or per chlorophyll. Comparison of the rate in terms of only one unit may be misleading since the percentage dry weight per fresh weight, and the percentage chlorophyll per dry weight varies from plant to plant and indeed (as discussed in Chapter 4) according to the age of the leaf. For comparison with the activity of isolated enzyme reactions the most convenient unit is that of μl./mg. dry weight/hour, i.e. the Q_{O_2} value which since oxygen is evolved in photosynthesis should be written as $-Q_{O_2}$. When this value is referred to chlorophyll content, i.e. μl./mg. chlorophyll/hour, it is called the $Q_{O_2}^{\mathrm{Chl.}}$ Maximum observed rates of photosynthesis expressed in these different units are given in the table on page 152.

Thus it can be seen that in *Sambucus* 1 mg. chlorophyll corresponds to 118 mg. dry weight or 450 mg. fresh weight. In *Chlorella* 1 mg. chlorophyll corresponds to 20 mg. dry weight or 100 μl. cell volume.

Plant	Per hour						
	mg. CO_2				µl. CO_2		
	mg. fresh weight	mg. dry weight	mg. chlorophyll (assimilation number)	100 cm.^{3} leaf area	mg. fresh weight	mg. dry weight Q_{CO_2}	mg. chlorophyll $Q^{Chl.}_{CO_2}$
Sambucus nigra							
Green variety	0·0146	0·053	6·6	34	—	26·5	3,300
Yellow variety	0·0088	0·047	117	18	—	23·5	58,500
Helianthus annuus	0·0230	0·134	14·0	80	—	67	7,000
	mg. O_2				µl. O_2		
					µl. cell volume	Q_{O_2}	$Q^{Chl.}_{O_2}$
Chlorella pyrenoidosa	0·06	0·30	6·0	—	30	150	3,000

(Data for leaves from Willstätter and Stoll, for *Chlorella* from our own laboratory.)

REFERENCES

A brief list of references of work referred to in the text is given below. A very comprehensive discussion of most aspects of photosynthesis can be found with the appropriate references in the monograph—

Photosynthesis, by E. I. Rabinowitch in three volumes. Published by Interscience Publishers, New York. Vol. I, 1945; Vol. II, 1951; Vol. III in press.

The Annual Review of Plant Physiology may also be consulted for reviews of current work on photosynthesis and related topics.

ARNON, D. I. (1951). Extracellular photosynthetic reactions. *Nature*, **167**, 1008.

ARNON, D. I. (1952). Glyceraldehyde phosphate dehydrogenase of green plants. *Science*, **116**, 635.

AXELROD, B., & JANG, R. (1953). Preparation and properties of phosphoriboisomerase. *Federation Proc.*, **12**, 172.

BANDURSKI, R. S., & GREINER, C. M. (1953). The enzymic synthesis of oxalacetate from phosphoryl-enolpyruvate and carbon dioxide. *J. Biol. Chem.*, **204**, 781.

BARKER, H. A. (1935). Photosynthesis in diatoms. *Arch. Mikrobiol.*, **6**, 141.

BLACKMAN, F. F. (1895). Experimental researches on vegetable assimilation and respiration. II: On the path of gaseous exchange between aerial leaves and the atmosphere. *Phil. Trans. Roy. Soc. Lond., B*, **186**, 503.

BLACKMAN, F. F. (1905). Optima and limiting factors. *Ann. Bot.*, **19**, 281.

BLACKMAN, F. F., & SMITH, A. M. (1911). On assimilation in submerged water plants and its relation to the concentration of carbon dioxide and other factors. *Proc. Roy. Soc. Lond., B*, **83**, 389.

BRIGGS, G. E. (1922). Experimental researches on vegetable assimilation and respiration. XVI: The characteristics of subnormal photosynthetic activity resulting from deficiency of nutrient salts. *Proc. Roy. Soc. Lond., B*, **94**, 20.

BRIGGS, G. E. (1929). The energetic efficiency of photosynthesis in green plants. *Proc. Roy. Soc. Lond., B*, **105**, 1.

BRIGGS, G. E. (1935). Photosynthesis in intermittent light, in relation to current formulations of the principles of the photosynthetic mechanism. *Biol. Rev.*, **10**, 460.

BRIGGS, G. E. (1941). Photosynthesis in intermittent illumination. *Proc. Roy. Soc. Lond., B*, **130**, 24.

BRIGGS, G. E., & WHITTINGHAM, C. P. (1952). Factors affecting the rate of photosynthesis of *Chlorella* at low concentrations of carbon dioxide. *New Phytol.*, **51,** 236.

BROWN, H. T., & ESCOMBE, F. (1905). Researches on some of the physiological processes of green leaves with special reference to the interchange of energy between the leaf and its surroundings. *Proc. Roy. Soc. Lond., B*, **76,** 29.

BROWN, A. H. (1953). The effect of light on respiration using isotopically enriched oxygen. *Amer. J. Bot.*, **40,** 719.

BROWN, A. H., FAGER, E. W., & GAFFRON, H. (1949). Kinetics of photochemical intermediates in photosynthesis. In FRANCK, J., and LOOMIS, W. E. *Photosynthesis in Plants*, Chap. 20.

BURK, D., & WARBURG, O. (1951). Ein Quanten Reaktion und Kreisprozess der Energie bei der Photosynthese. *Die Naturwissenschaften*, **37,** 560; *Z. Naturforsch.*, **66,** 12.

BURSTROM, H. (1943). Photosynthesis and the assimilation of nitrate by wheat leaves. *Ann. Roy. Agr. Coll. Sweden*, II, 1.

CALVIN, M. (1949). The path of carbon in photosynthesis. *J. Chem. Educ.*, **26,** 639.

CALVIN, M. *et al.* (1951). Carbon dioxide assimilation in plants. *Soc. Exp. Biol., Photosynthesis Symposium* 284.

CALVIN, M., & MASSINI, P. (1952). The path of carbon in photosynthesis. XX. *Experientia*, **8,** 445.

CADVIN, M. (1954). The formation of phosphoglyceric acid from ribulosediphosphate and carbon dioxide in presence of cell-free extract. Personal communication.

CLENDENNING, K. A., & EHRMANTRAUT, H. (1950). Photosynthesis and Hill reactions by whole *Chlorella* cells in continuous and flashing light. *Arch. Biochem.*, **29,** 387.

COOKSON, G. H., & RIMINGTON, C. (1953). Porphobilinogen; chemical constitution. *Nature*, **171,** 875.

CORI, O., & RIPMANN, F. (1952). The primary oxidation product of enzymatic glucose-6-phosphate oxidation. *J. Biol. Chem.*, **194,** 417.

DAVENPORT, H. E., & HILL, R. (1951). The preparation and some properties of cytochrome *f*. *Proc. Roy. Soc. Lond.*, **139,** 327.

DICKENS, F. (1938). Oxidation of phosphohexonate. *Biochem. J.*, **32,** 1626.

DIXON, M. (1950). *Multienzyme systems*. Cambridge University Press.

DUTTON, H. J., & MANNING, W. M. (1941). Evidence for carotenoid-sensitized photosynthesis in the diatom *Nitschia closterium*. *Amer. J. Bot.*, **28,** 516.

DUYSENS, L. N. M. (1952). Ph.D. Thesis, Utrecht.

EMERSON, R., & ARNOLD, W. (1932). A separation of the reactions in photosynthesis by means of intermittent light. *J. Gen. Physiol.*, **15,** 391.

EMERSON, R., & LEWIS, C. M. (1943). The dependence of the quantum field of *Chlorella* photosynthesis on wavelength of light. *Amer. J. Bot.*, **30,** 165.

EMERSON, R., & LEWIS, C. M. (1942). The photosynthetic efficiency of phycocyanin in *Chroococcus*. *J. Gen. Physiol.*, **25**, 579.

FAGER, E. W. (1952). Photochemical carbon dioxide fixation by a cell-free system. *Arch. Biochem.*, **41**, 383.

FOGG, G. E. (1953). *The metabolism of algae*. Methuen, London.

FÖRSTER, TH. (1949). Experimentelle und theoretische untersuchung des zwischenmolekularen übergangs von elektronenanregungsenergie. *Z. Naturforsch.*, **4a**, 321.

FRANCK, J. (1949). The relation of the fluorescence of chlorophyll to photosynthesis. In FRANCK, J., and LOOMIS, W. E. *Photosynthesis in Plants*, Chap. 16.

FRANCK, J., & FRENCH, C. S. (1941). Photoxidation processes in plants. *J. Gen. Physiol.*, **25**, 309.

FRANCK, J., FRENCH, C. S., & PUCK, T. T. (1941). The fluorescence of chlorophyll and photosynthesis. *J. Phys. Chem.*, **45**, 1208.

FRANCK, J., & HERZFELD, K. F. (1941). Contribution to a theory of photosynthesis. *J. Phys. Chem.*, **45**, 978.

FRANCK, J., PRINGSHEIM, P., & LAD, D. T. (1945). Oxygen production by anaerobic photosynthesis of algae measured by a new micromethod. *Arch. Biochem.*, **7**, 103.

FRENCH, C. S., & YOUNG, V. K. (1953). The fluorescence spectrum of red algae and the transfer of energy from phycoerythin and phycocyanin to chlorophyll. *J. Gen. Physiol.*, **35**, 873.

FRENKEL, A. W. (1941). Photosynthesis with radio active carbon and the distribution of the intermediate products in the plant cell. *Plant Physiol.*, **16**, 654.

GABRIELSEN, E. K. (1935). Die Kohlensäureassimilation der Laubblätter in verschiedenenSpektralgebieten. *Planta*, **23**, 474.

GABRIELSEN, E. K. (1948). Threshold value of carbon dioxide concentration in photosynthesis of foliage leaves. *Nature, Lond.*, **161**, 138.

GAFFRON, H. (1927). Sauerstoffübertragung durch chlorophyll und das photochemische Aquivalent gesetz. *Ber. deutsch. Chem. Gesell.*, **60**, 755.

GAFFRON, H. (1944). Photosynthesis, photoreduction and dark reduction of carbon dioxide in certain algae. *Biol. Rev. Cambridge Phil. Soc.*, **19**, 1.

GIBBS, M. (1952). Triosephosphate dehydrogenase and glucose-6-phosphate dehydrogenase in the pea plant. *Nature*, **170**, 164.

GLOCK, G. E. (1952). The formation and breakdown of pentose-phosphates by liver fractions. *Biochem. J.*, **52**, 575.

GRANICK, S. (1949). The chloroplasts: Their structure, composition and development. Chap. 5 in *Photosynthesis in Plants*.

GRANICK, S. (1950). The structural and functional relationships between heme and chlorophyll. *Harvey Lectures*, Ser. **44**, 220.

HARDER, R. (1921). Kritsche versuche zu Blackmans Theorie der bergreuzenden Faktoren bei der Kohlensäureassimilation. *Jb. Wiss. Bot.*, **60**, 531.

HAXO, F. T., & BLINKS, L. R. (1950). Photosynthetic action spectrum of marine algae. *J. Gen. Physiol.*, **33**, 389.

HEATH, O. V. S. (1951). Assimilation by green leaves with stomatal control eliminated. In *Carbon Dioxide Fixation and Photosynthesis. Fifth Symposium of the Soc. Exp. Biol.* (London), C.U.P., 1951.

HERBERT, D. (1951). In *Symposia of Soc. Exp. Biol.* (London), p. 52.

HILL, R., & SCARISBRICK, R. (1940). Production of oxygen by illuminated chloroplasts. *Nature*, **146**, 61.

HILL, R., & SCARISBRICK, R. (1940). The reduction of ferric oxalate by isolated chloroplasts. *Proc. Roy. Soc. Lond., B.*, **129**, 238.

HILL, R., & WHITTINGHAM, C. P. (1953). The induction phase of photosynthesis in *Chlorella. New Phytol.*, **52**, 133.

HOLT, A. S., & FRENCH, C. S. (1948). Isotopic analysis of the oxygen evolved by illuminated chloroplasts. *Arch. Biochem.*, **19**, 429.

HORECKER, B. L. (1953). A new pathway for the oxidation of carbohydrate. *Brewers' Digest*, **28**, 214.

HORECKER, B. L., & SMYRNIOTIS, P. Z. (1951). Phosphogluconic acid dehydrogenase from yeast. *J. Biol. Chem.*, **193**, 371.

LARSEN, H., YOCUM, C. S., & VAN NIEL, C. B. (1952). On the energetics of the photosynthesis in green sulphur bacteria. *J. Gen. Physiol.*, **36**, 161.

LEMBERG, R., & LEGGE, J. W. (1949). *Haematin compounds and bile pigments*. Interscience Publ., New York.

LIPMANN, F. (1941). Metabolic generation and utilization of phosphate bond energy. *Adv. Enzymol.*, **1**, 99.

MACDOWALL, F. D. H. (1949). The effects of some inhibitors of photosynthesis upon the photochemical reduction of a dye by isolated chloroplasts. *Plant Physiol.*, **24**, 462.

MACKINNEY, G. (1941). Absorption of light by chlorophyll solutions. *J. Biol. Chem.*, **140**, 315.

MASKELL, E. J. (1928). The relation between stomatal opening and assimilation. *Proc. Roy. Soc. Lond.*, *B*, **102**, 488.

MATTHAEI, G. L. C. (1904). Experimental researches on vegetable assimilation and respiration. III: On the effect of temperature on carbon dioxide assimilation. *Proc. Roy. Soc. Lond.*, *B*, **197**, 47.

MCALISTER, E. D., & MYERS, J. E. (1940). The time course of photosynthesis and fluorescence observed simultaneously. *Smithsonian Inst. Pub. Misc. Coll.*, **99**, (6).

MEHLER, A. H., & BROWN, A. H. (1952). Studies on reactions of illuminated chloroplasts. *Arch. Biochem.*, **38**, 367.

MILLERD, A., & BONNER, J. (1953). Oxidative phosphorylation by plant mitochondria. *Arch. Biochem.*, **42**, 135.

NEUBERGER, A., & SCOTT, J. J. (1953). Aminolaevulinic acid and porphyrin biosynthesis. *Nature*, **172**, 1093.

O'KANE, D. J., & GUNSALUS, K. (1948). Pyruvic acid metabolism —factor required for oxidation by *Streptococcus faecalis*. *J. Bact.* **56**, 499.

PARDEE, A. B., SCHACHMAN, M. K., & STANIER, R. Y. (1952). Chromatophores of *Rhodospirillum rubrum*. *Nature*, **169**, 282.

PENMAN, H. L., & SCHOFIELD, R. K. (1951). Some physical aspects of assimilation and transpiration. In *Carbon Dioxide Fixation and Photosynthesis*. Vol. 5, *Soc. Exp. Biol. Symposia*.

PORRETT, D., & RABINOWITCH, E. (1937). Reversible bleaching of chlorophyll solutions. *Nature, Lond.*, **140**, 321.

ROTHEMUND, P. (1944). Chlorophyll. In *Medical Physics*, Otto Glasser, Chicago.

RUBEN, S., KAMEN, M. D., & HASSID, W. Z. (1940). Photosynthesis with radioactive carbon. *J. Amer. Chem. Soc.*, **62**, 3443.

SHEMIN, D., & RUSSELL, C. S. (1953). δ-aminolaevulinic acid and its role in the biosynthesis of porphyrins and purines. *J. Amer. Chem. Soc.*, **75**, 4873.

SMITH, J. H. C. (1949). Products of photosynthesis. In FRANCK, J., & LOOMIS, W. E. *Photosynthesis in Plants*, Chap. 3.

SMITH, J. H. C., FRENCH, C. S., & KOSKI, V. M. (1952). The Hill reaction: development of chloroplast activity during greening of etiolated barley leaves. *Plant Physiol.*, **27**, 212.

STREHLER, B. L., & ARNOLD, W. (1951). Light production in green plants. *J. Gen. Physiol.*, **34**, 809.

STREHLER, B. L. (1951). The luminescence of isolated chloroplasts. *Arch. Biochem.*, **34**, 239.

STREHLER, B. L. (1953). Firefly luminescence in the study of energy mechanisms. *Arch. Biochem.*, **43**, 67.

SVEDBERG, T., & KATSURAI, T. (1929). The molecular weights of phycocyanin phycoerythrin from *Porphyra tenera* and of phycocyanin from *Aphanizomenon Flos Aquae*. *J. Amer. Chem. Soc.*, **51**, 525.

TANADA, T. (1951). The photosynthetic efficiency of carotenoid pigments in *navicula minima*. *Amer. J. Bot.*, **38**, 276.

TOLMACH, L. J. (1951). Triphosphophyridine nucleotide and chloroplasts. *Arch. Biochem.*, **33**, 120.

VAN DEN HONERT (1930). Carbon dioxide assimilation and limiting factors. *Rec. trav. bot. neerl.*, **27**, 149.

VAN NIEL, C. B. (1941). The bacterial photosyntheses and their importance for the general problem of photosynthesis. *Adv. Enzymol.*, **1**, 263.

VAN NIEL, C. B. (1949). The comparative biochemistry of photosynthesis. In FRANCK, J., & LOOMIS, W. E. *Photosynthesis in Plants*, Chap. 22.

VENNESLAND, B., & CONN, E. (1952). Carboxylating enzymes in plants. *Ann. Rev. Pl. Physiol.*, **3**, 307.

VISHNIAC, W., & OCHOA, S. (1951). Photochemical reduction of pyridine nucleotides by spinach grana and coupled carbon dioxide fixation. *Nature*, **167**, 768.

VISHNIAC, W., & OCHOA, S. (1952). Phosphorylation coupled to photochemical reduction of pyridine nucleotides by isolated chloroplasts. *J. Biol. Chem.*, **198**, 501.

VOERKEL, S. M. (1933). Untersuchungen über die Phototaxis der chloroplasten. *Planta*, **21**, 156.

VOGLER, K. G., & UMBREIT, W. W. (1942). The nature of the energy storage material active in the chemosynthetic process. *J. Gen. Physiol.*, **26**, 157.

WARBURG, O. (1919). Über die Geschwindigkeit der photochemischen Kohlensaürezersetzung in lebenden Zehlen. *Biochem. Z.* (1919), **100**, 230; and *Biochem. Z.* (1920), **103**, 188.

WARBURG, O., & CHRISTIAN, W. (1939). Isolierung und Kristallisation des Proteins des oxydierenden Garüngsferment. *Biochem. Z.*, **303**, 40.

WARBURG, O., & LUTTGENS, W. (1946). Photochemische Reduktion des Chinons in grunen zellen und granula. *Biochemiya*, **11**, 303.

WARBURG, O., & NEGELEIN, E. (1920). Über den Energieumsatz bei der Kohlensäureassimilation. *Z. Physikal. Chem.*, **102**, 236.

WARBURG, O., & NEGELEIN, E. (1922). Über die Reduktion der Salpetersäure in grünen Zellen. *Biochem. Z.*, **110**, 66.

WASSINK, E. C., KATZ, D., & DORRESTEIN (1941). On photosynthesis and fluorescence of bacteriochlorophyll in Thiorhodaceae. *Enzymologia*, **10**, 285.

WASSINK, E. C., & KERSTEN, J. A. H. (1946). Observations sur la spectre d'absorption et sur la rôle des carotenoids dans la photosynthèse des diatomées. *Enzymologia*, **12**, 3.

WASSINK, E. C., VERMEULEN, D., REMAN, G. H., & KATZ, E. (1938). On the relation between fluorescence and assimilation in photosynthesizing cells. *Enzymologia*, **5**, 100.

WELLER, S., & FRANCK, J. (1941). Photosynthesis in flashing light. *J. Phys. Chem.*, **45**, 1359.

WHATLEY, F. R. (1951). Coenzymes in plants. *New Phytol.*, **50**, 244.

WHITTINGHAM, C. P. (1952). Inhibition of photosynthesis by cyanide. *Nature*, **169**, 838.

WILLSTÄTTER, R., & STOLL, A. (1913). *Untersuchungen über chlorophyll.* Julius Springer (Berlin).

WILLSTÄTTER, R., & STOLL, A. (1918). *Untersuchungen über die Assimilation der Kohlensäure.* Julius Springer (Berlin).

WOOD, H. G. (1946). The fixation of carbon dioxide and the interrelationships of the tricarboxylic acid cycle. *Physiol. Rev.*, **26**, 198.

ZSCHEILE, F. P., & COMAR, C. L. (1940). Influence of preparative procedure on the purity of chlorophyll components as shown by absorption spectra. *Bot. Gaz.*, **102**, 463.

ZSCHEILE, F. P., WHITE, J. W., BEADLE, B. W., & ROACH, J. R. (1942). The preparation and absorption spectra of five pure carotenoid pigments. *Plant Physiol.*, **17**, 331.

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